

10 April 1980

Eleventh hour for biotechnology in Britain

WHETHER or not the National Enterprise Board decides to, or is allowed to, set up a biotechnology company, the Spinks report published last week was right to have suggested that the Board consider the possibility (see page 502). There is little doubt that Britain has long been in need of companies prepared to risk investment in biotechnology. The only question now is whether it is too late.

As far back as 1975 it was obvious to a certain number of UK scientists that there was considerable commercial potential in new techniques emerging from molecular and cellular biology. In particular it was recognized that the technique of recombinant DNA might be used to harness bacterial and other cells to the production of pharmaceutically active polypeptides. Gene manipulation was also seen to have much to offer in the improvement of strains of microorganisms employed in fermentation and waste disposal. Bacterially cloned genes could also be foreseen as valuable in the diagnosis and therapy of congenital diseases. Equally there was early, if limited, recognition of the commercial potential of hybridoma cells which produce single antibodies — which are of considerable value in radioimmunoassays and other medical diagnostic techniques.

It was with some chagrin that scientists in Britain, including those responsible for some of the key discoveries, watched entrepreneurs in the US start up a series of small venture capital businesses aimed to cash in on the new techniques, whereas nothing equivalent emerged in the UK. It still has not.

Nor, with a few exceptions, have established UK industries been quick off the mark to exploit the new techniques. Most companies have seemed content to bide their time rather than to fund research that may have looked promising but whose value would not be proven without long term investment. In the event, progress abroad has been much faster than was anticipated. Although real fortunes, as opposed to paper ones, are not yet being made from the new biotechnology, there can be no doubt that they will be. And yet there is still a distinct lack of interest from UK industry.

With neither established nor new companies to fund and benefit from UK scientific expertise, universities and organisations such as the Medical Research Council and the Agricultural Research Council find it difficult to exploit commercially the discoveries of their scientists. Could their requirements be met by a publically financed company?

Any such proposal will meet three hurdles. The first is time; is it too late to set up such a company? Here the answer must be a qualified no. It is probably too late to think in terms of producing hormones and vaccines by means of genetically-manipulated bacteria; those are first generation applications already far advanced. But there is no shortage of second generation applica-

tions, particularly those involving fungal, mammalian and plant cells, with which a newly formed company might hope to leapfrog the early starters.

The second hurdle is manpower. While British industry and entrepreneurs have been dallying, many of the academic scientists have become involved with US-based, or US-financed, venture capital companies. Other British scientists have resisted all approaches and will probably continue to do so. It is therefore dangerously late to be recruiting scientists to the cause. Nevertheless there does appear to remain sufficient untapped expertise, particularly in the laboratories of those research councils most keen to see a company founded, to make the project worth pursuing.

The third hurdle is the Conservative government which, particularly with its present leaders, strongly favours private over public enterprise. But faced with a demand unfulfilled by the private sector no government should stand endlessly on principle.

If all three hurdles can be jumped, there remains the question of how best to set up a public company. The obvious way, as recommended in the Spinks report, is through the National Enterprise Board (NEB) and/or the National Research Development Corporation (NRDC). Although the NRDC already has some expertise and experience in the patenting and licencing of biotechnological processes, there are drawbacks to its further involvement. In the first place whereas it commonly gives a half share of royalties to any university from which a patented discovery has emerged, it is constitutionally unable to pass royalties back to research council laboratories. Secondly it is willing only to fund research with a clear commercial potential. Thirdly, and despite its having taken out such important patents as those on cephalosporins and interferon (unfortunately time-expired except in the US, where it runs until 1989), the NRDC has not consistently spotted the winners. One glaring mistake is its 1975 decision that hybridomas had no obvious commercial applications.

Given the drawbacks of the NRDC, there is a good case for the NEB to found a biotechnology company. The chief attraction of this proposal is simply that a new company, devoted to a relatively narrow venture, would be able to avoid most of the problems associated with the NRDC. Clearly the success of such a company would hinge crucially on the calibre of its staff, their ability to pick areas in which they could mount an effective challenge to those companies already in the business, and the degree to which productive links could be built and maintained with academic scientists.

None of this will be easy. It will become even less easy as time passes. Therefore if, as we believe, the NEB should start a biotechnology company in the UK it should do so now. □



United States

Budget cuts stop research growth

Delays in space science missions, construction of a heavy ion accelerator, and a scheme to improve university research facilities are among the victims of last week's US budget cuts. **David Dickson** reports

It could have been worse — and for a time it looked as if it was going to be. But when President Carter announced last week where the axe was to fall in his attempt to balance the federal budget, it became clear that the growth enjoyed by basic research for the past few years has, at least temporarily come to a halt. Indeed the first indications are that the cuts, reducing expansion to below the expected inflation level, will mean a net contraction in overall funding.

Hardest hit will be the National Science Foundation, which had expected a 17.7% growth in its budget in 1981, but has had this reduced by \$74 million to an 11% increase. Close behind is the National Aeronautics and Space Administration, although the space shuttle programme has been protected from cuts, largely because of its commercial and military significance.

In other agencies the impacts have been less severe. The Department of Energy, for example, has had the growth in its basic research budget cut from 13.4% to 12.2%. And at the National Institutes of Health, where possible cuts of up to \$500 million had initially been discussed (largely because it is one of the few areas of discretionary spending in the health budget), it was finally agreed to reduce the 1981 basic research budget by only \$35 million out of \$1,704 million.

Dr Frank Press, Director of the Office of Science and Technology Policy, told *Nature* last week that in deciding where the President's original budget proposals for 1981 should be reduced, it was decided to aim primarily for demonstration and development projects rather than basic research support.

"New initiatives were slowed down but not dropped if they were philosophically important to us" said Dr Press.

Some of the more detailed cuts are: ●NASA: total research and development budget reduced from \$5.7 to \$5.5 billion. The space science budget, previously planned to rise from \$600.9 million to \$668 million, will now fall to \$561 million.

In addition to a two-year delay in the Solar Polar Mission, future experiments in physics, astronomy, life sciences and environmental observation planned for flight on Spacelab missions will be delayed for one to two years.

These cuts will leave unaffected two new starts proposed for the 1981 budget, namely the Gamma Ray Observatory, and the National Oceanic Satellite System. Nor

will the cuts effect either the Galileo Mission or the space telescope, both initially under threat when possible cuts of up to \$760 million were being discussed.

However funding for the Earth Radiation Experiment, NASA's proposed contribution to the National Climate Plan, will be delayed for one year. And the agency will also be cutting back on cosmic ray research, as well as terminating relativity research and the analysis of data from earlier pioneer missions (although the latter will be kept for future analysis).

●Department of Energy: the high energy physics programme will be cut by \$4

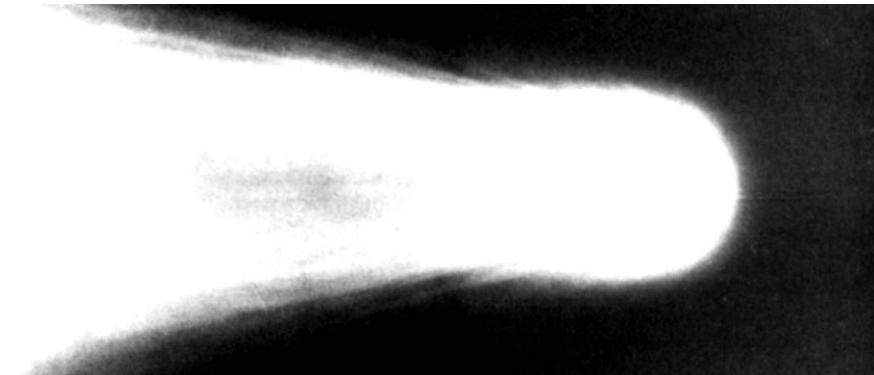
million from its original proposed level of \$359 million. This cut will be absorbed at Brookhaven, where the construction of Isabelle is already experiencing delay due to problems with the superconducting magnets.

A similar sum is being cut from the nuclear physics budget by deferring for one year construction of the Heavy Ion Beam Argonne tandem accelerator system.

In addition, \$3million inserted into the budget to support the setting up of advanced energy research centres at leading US universities (an idea proposed only last autumn by Dr David Saxon, of the University of California) has been dropped.

●National Science Foundation: within the NSF two strategies have been pursued. Firstly to preserve as much as possible of core responsibilities for basic research.

Delay for space mission with Europe



Halley's comet: delays to the solar polar mission mean hope for a cometary mission for Europe

AMONG the organizations to be affected by the US budget cuts is the European Space Agency, which is collaborating with NASA on a project to send two spacecraft in complementary polar orbits around the Sun, known as the International Solar Polar Mission.

The Carter administration, which included the mission as a new start in last year's budget submission to Congress, is now proposing to save \$43 million in 1981 (compared to \$83 million proposed in January) by delaying the launch of the mission from 1983 to 1985.

The delay will cause serious programming problems for ESA, whose space science projects are already on a tight schedule, and which last summer signed a \$56 million contract with Dornier to construct the European spacecraft by 1983.

The agency now has to decide whether to allow construction to be completed as planned, and subsequently put the spacecraft in mothballs; or to slow down construction work, hoping that the money

saved can be absorbed by other projects and will not have to be returned at the end of the year.

Either strategy will make the project more expensive than when it was approved.

This particular cloud may, however, prove to have a silver lining. For some of the money freed from the ISPM might be available to support a fly-by mission to the comet Halley, which would have to be launched at the end of 1984.

After NASA had informed ESA in December that it was unlikely to be able to support a joint mission to Halley and the comet Tempel 2 because funding for a solar electric propulsion system had been deleted by the Office of Management and Budget, ESA's scientific programme committee agreed last month that it would be prepared to consider a European solo mission, providing the costs are under 80 million accounting units (about \$110 million).

Detailed proposals for such a mission will be considered when the committee meets again in August. □

And secondly to maintain the direction of new initiatives introduced into the 1981 budget as a result of last year's domestic policy review of industrial innovation, even though at a reduced level.

In areas of project support, each field will be scaled downwards, although the emphasis will be maintained on shifting the balance of support back to physics and engineering from the life sciences. Thus the total for mathematical and physical sciences will be reduced by \$4.4 million (leaving a 14.5% increase over 1980), and for biological and behavioural sciences by (\$4.1 million, leaving a 6.8% increase).

The increase in funding for university-industry cooperative research projects will be reduced by \$5.1 million, from \$13 to \$7.9 million above this year's level of \$7 million. And support for small business innovation and industrial technology, initially scheduled to be raised from \$7.3 million to \$18.2 million, will now only increase to \$9.2 million.

Another new initiative to be cut back is the Ocean Margins Drilling Project. This is to be jointly funded with the oil industry, and had been scheduled to receive \$10 million in 1981, but the figure will be cut back to \$5 million.

More money has been saved by putting off detailed design studies of a 25-metre diameter millimeter wave telescope, which would have been scheduled for funding in 1982, as well as plans to purchase a new coastal research ship.

The NSF has also decided to defer a proposal to award \$14.3 million on a matching grant basis for improving university research facilities. There will also be a \$10 million cut in science education programmes.

Finally, the Foundation is cutting back on its joint scientific programmes with both the USSR and the China (the latter introduced for the first time in 1931). In particular, a programme with Soviet scientists on chemical catalysis, and a working group in scientific and technical information, will both be terminated, and these, together with cutbacks in exchanges through the National Academy of Science, will reduce the US/USSR budget from \$3.2 to \$1 million. Scientific cooperation with China will have its support reduced from a proposed \$2 million to \$1.5 million.

● **Defense Department:** the Department of Defense is the one agency to have escaped significant budget cuts. Precise figures have yet to be agreed, but it is expected that the department's original proposal will be cut back by between \$10 and \$20 million. This will still permit an increase in military support for basic research of about 15% — well above the expected rise in costs.

The big question now is how Congress will react. In general the cuts from its original budget proposals recommended by the administration are in line with those supported by Congressional budget committees, but there could be more surprises in store. □

Energy choices: the cultural assumptions

THE debate surrounding the report of the National Academy of Sciences Committee on Nuclear and Alternative Energy Systems (CONAES) continues to reveal as much about different perspectives on the energy problem as it throws light on possible solutions.

Latest contribution to this debate is the report of a panel set up by the committee to study the "lifestyle" implications of different projected energy scenarios. Published by the Academy last week, the report carries the emphatic message that "energy is a social, not a technological issue".

A society's demand for energy must be seen as part of its general organisation of preferences. Yet in many discussions of the factual basis of energy problems, the cultural and social contexts tend to be left implicit; and research on social and cultural factors influencing energy decisions has consequently received "remarkably little attention".

"We know something about the technology of energy, but much less about the agents: the experts, the interest groups, the public," says the report, which recommends that more research is needed on the social organisation of energy experts.

"Do nuclear physicists and engineers, for example, dominate the latecoming biologists, health physicists and social scientists?" it asks. "What is the relationship between the experts who estimate the probabilities of harmful events and those who assess their consequences?"

The panel which produced the report was chaired by Dr Laura Nader Professor of Anthropology at the University of California, Berkeley. In publishing it, the Academy points out that it is intended as a "supporting paper" to the full CONAES report, and that it has not gone through the "normal critical review" for such reports — although adding that it has been "subjected to a thorough and expert peer review for accuracy, consistency and clarity."

The bulk of the report concentrates on some of the possible implications of two future energy scenarios that might be expected in the year 2010. The first assumes a level of energy consumption in the US of 71 quads, equal to estimates of consumption in 1975 when the study was started.

This level of energy demand, says the report, could be achieved without any significant changes in attitudes, and would still involve increases in amenities, with improvements "roughly consistent with those that have occurred in recent decades."

The second scenario is based on the assumption that society agrees to cut its consumption to 53 quads — and is offered by the panel not as a prediction of what is likely to happen, but as a way of focusing on the relationship between energy demand and cultural values.

This scenario, says the panel, implies a significant shift in attitudes, including a decentralisation of work, and high value being placed on thrift and self-reliance. In what it characterises as a "high technology, low energy consumption" society, the aim would not be to turn back the clock, but to develop new technologies — such as advanced automobiles, or microprocessor building and process control techniques — aimed at improving the quality of life under the new energy constraint.

Pointing out that societies such as Sweden's already exist on much lower energy consumption than the US, the panel suggests that its scenario would be feasible, and would reflect the needs of a participatory democracy which might be challenged by increasingly centralised technological systems (such as those involved in the use of nuclear power).

"The trends toward tightly meshed technological systems characteristic of the 1970s are reversed in the 53 quad society, increasing the likelihood that most of the system can survive if a part of it is severely damaged," the report says.

In its conclusion, which stresses the need to integrate long-term energy planning with other kinds of social planning, the panel emphasises that there is nothing improper in the fact that political and social decisions about resource use extend beyond the market place and are influenced by subjective values and judgments.

"What is alarming is that experts often pretend to be able to perform objective analyses on energy systems or energy users because they are unwilling to acknowledge the values on which their decisions or analyses were made," it says.

Perhaps unsurprisingly, few of the more outspoken conclusions were included in the final report of the full CONAES committee. And this itself has provoked a reaction from Dr Nader, who says that her panel was told that its work "was not quantitative enough", and should include "more tables and less prose."

"The criticisms raised old disagreements about the use of qualitative and quantitative methods and illustrate how difficult it is in general for many scientists to incorporate findings from disciplines which could contribute to more intelligent discussions of unbounded problems" says Dr Nader. □

UK biotechnology

R&D attitudes wrong, says report

The "Spinks report" on the future of biotechnology in the UK was finally published last week. **Robert Walgate** reports

"THERE is a clear pre-development gap in R&D funding in Britain" Dr Alfred Spinks, chairman of a working party whose report* on UK biotechnology had just been published told a press conference last week. There is too little adventurousness, "a lack of gambling money". This is what is needed now for biotechnology in the UK, though it was clear that "one cannot transform the UK into an entrepreneurial state like California".

Dr Spink's report, prepared by the seven-man working party, says that "the present structure of public and private support for R&D is not well-suited to the development of a subject like biotechnology which, at the moment, straddles the divisions of responsibility both among government departments and among research councils and the arbitrarily defined fields of applied and fundamental research".

The recommendations and criticisms in the report are substantially the same as those included in a draft version detailed in *Nature* on 24 January (page 324). A joint committee for biotechnology should be set up to coordinate the biotechnical work of the five research councils, and a parallel interdepartmental steering committee set up for government departments.

Research councils should double their present commitment to biotechnology to a level of at least £3 million a year, and government departments should commit some £2.5 million. The National Enterprise Board and National Research Development Corporation should consider setting up a "research-oriented biotechnology company" with £2 million a year for five years.

John Ashworth, Chief Scientist at the Cabinet Office, who coordinated the working party, said "after five years we would expect industry to come in and invest in the company. If they don't, the venture will have failed."

"People are the main problem, not money" said Ashworth. The estimated spending on a biotechnical firm had included £1 million a year overheads, plus 50 scientists at £10,000 salary plus £10,000 research expenses. The 50 people could be found without difficulty, despite the increasing recruitment to foreign firms, thought Dr Spinks. "And we should also look abroad for talent".

The report supports the expansion of centres of excellence in the subject in universities. "A minimum of 20 new teaching and research posts should be created over the next five years with a capital investment of around £2 million" for lab facilities, says the report, and careful attention should be paid to the

training of an appropriate workforce.

● Professor Brian Hartley, a member of the Spinks working party, said last week that it was too late to consider setting up a firm to exploit British biotechnical talent, as recommended in the report.

Professor Hartley is a British member of Biogen, a US and Canadian-funded genetic engineering contract research body on which the UK firm, code-named 'Greenfields' by the National Enterprise Board, might be modelled. Biogen announced the cloning of interferon in the bacterium *E. coli* a few weeks ago, and completed a laboratory in Geneva last December; and recently it has been recruiting new staff.

The problem a new UK-based firm would face would be to find enough good staff," said Hartley. "A month ago they were available. Now they've joined us."

Hartley would not give precise figures. But "a few tens" have been recruited for the Geneva laboratory, to be compared with the 50 required for the UK firm. □



*Biotechnology, HMSO, £3

Crucial NEB decision due today

If all goes according to plan, the UK National Enterprise Board (NEB) will today be taking a crucial decision on whether or not to seek ministerial approval to sink at least £10 million of public funds into British biotechnology.

The idea that the NEB should become involved in biotechnology receives impetus from last week's publication of the Spinks report which recommends that public funds be used to set up a research-orientated biotechnology company in Britain. However, the NEB were already investigating the possibility last summer. And last December, Mr Benjamin Lewin, editor of the journal *Cell*, was in the UK sounding out the opinion of British scientists on behalf of the NEB.

Although the details of the proposal are unclear, it is believed to favour both the establishment of a small specialist company and the funding of promising research in academic laboratories. In that way it might be possible to make the most of the proposed investment of £10 million, over a five-year period.

If the NEB decides to proceed with the British proposal, it is almost certain to have to seek approval from the Secretary of State, Sir Keith Joseph. Strictly speaking, the NEB would not need to do this because it was delegated the authority to invest up to £10 million by the Labour government which set it up in 1975. The Conservative government is less well disposed towards the concept of the NEB, (particularly

because it was often used to rescue the lame ducks of British industry) and is in the process of limiting its role and reducing to £5 million the sum it can invest without ministerial approval.

Likely recipients of any funds made available by an NEB-owned company would include the Agricultural Research Council's Plant Breeding Research Institute, the Imperial Cancer Research Fund laboratory in London and such Medical Research Council (MRC) laboratories as the Cell Immunology Unit in Oxford and the Laboratory of Molecular Biology in Cambridge. Within each of these laboratories there is considerable expertise on two of the techniques which underpin the current wave of biotechnology, namely genetic engineering through recombinant DNA techniques and monoclonal antibody production from hybrid cells.

Under pressure from the Treasury to take more seriously than it sometimes has done the possibility of commercially exploiting scientific discoveries made in its laboratories, the MRC has recently reminded its senior scientific staff of their obligation to draw to the Council's attention any results of commercial potential. In particular the MRC is interested in the distribution and marketing of monoclonal antibodies derived from hybrid cells by the technique devised in 1975 by Dr Caesar Milstein and Dr George Kohler in the MRC Laboratory

of Molecular Biology.

The potential market value of monoclonal antibodies and the lack of a satisfactory route for commercially exploiting them is well known to the MRC. At the end of February it asked its scientific staff to provide full information on the availability and characterisation of hybrid cell lines as they were produced. At the same time the MRC said that it was exploring the means by which such cells and the antibodies produced from them could be distributed and marketed in a coordinated way.

Questioned whether this represented a new departure in MRC policy, Dr James Gowans, MRC secretary, said that the policy was not new but that, under pressure from the Treasury, the possibilities of commercial exploitation were being looked at more aggressively. Many monoclonal antibodies produced in MRC laboratories are already being marketed through the small UK firm of Sera-Lab Ltd, with a proportion of the sales revenue being channelled back to the laboratories. But, said Gowans, the antibodies marketed by Sera-Lab tended to be those of marginal commercial value. For the development and marketing of antibodies of high commercial value either because of the anticipated volume of sales or because of the rarity of the cell hybrids from which the antibody was derived, Gowans would welcome the existence of a company of the kind that the NEB might start.

Peter Newmark

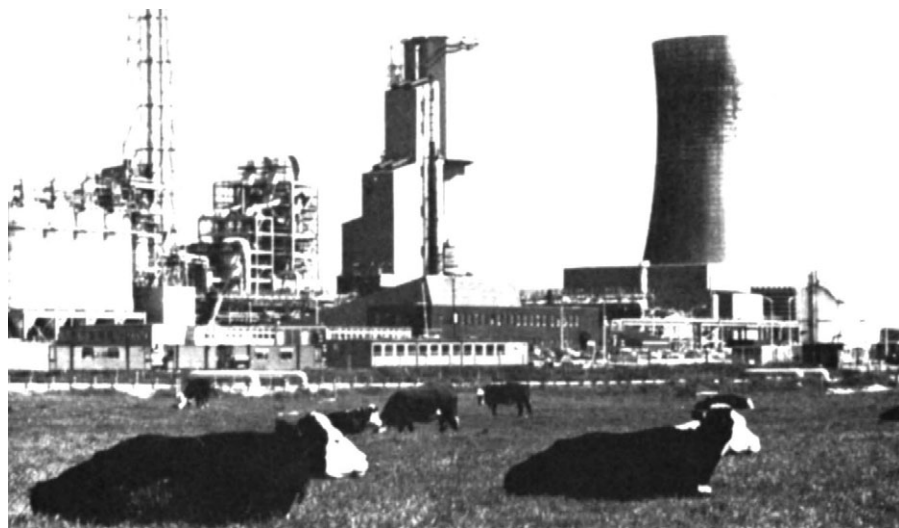
Spinks offers "too little"

THE chairman of Cetus Corporation, America's first and largest contract biotechnical enterprise, believes that £2 million a year for five years is far too little to invest in a new biotechnical firm — as envisaged in the Spinks report. Pressed as to what would be a reasonable investment, he suggested \$50 million over five years would be the minimum "if you are not to waste your cash".

The Cetus chairman, Dr Ronald Cape, was speaking at a conference on biotechnology in Rotterdam at the end of last month. He eschews government funding or work undertaken in universities outside Cetus's direct control. "We do our work in our labs" he said.

Returns will be long in coming, thinks Cape: no genuine profit for 5 to 10 years. Nevertheless, Cetus "is now investing millions of dollars of its own money, and comparable amounts on behalf of major company clients," in biotechnology.

New companies like Cetus will move biotechnology forward, thinks Cape, but new companies are not necessarily small. Cetus has 250 employees — "the largest collection of molecular biologists in the world" — and 18 projects in five industries, all with potential markets of billions of dollars. "Biotechnology is no longer cheap" says Cape. □



Two types of fermenter: cows outside ICI's single cell protein plant

Single cell protein organism improved

MICRO-ORGANISMS selected by the British firm Imperial Chemical Industries (ICI) to produce single cell protein (SCP) have been made more carbon-efficient by an application of recombinant DNA techniques, the firm announced last week.

ICI recently established the world's largest SCP fermenter — more than 50m high — using a methanol-consuming organism known as AS1. But the process has been under constant market pressure from agriculturally-produced soya-bean protein (both are used as animal feed supplements) and so ICI has been at pains to reduce the basic cost of its SCP. One obvious target was to improve the efficiency with which AS1 converts the costly methanol feedstock into protein.

Protein requires nitrogen, and AS1 gets its nitrogen from ammonia. But ICI discovered that AS1 assimilates the ammonia by an energy-inefficient pathway — one using the enzyme glutamate synthase to convert ammonia to glutamate. The energy for this comes ultimately from the oxidation of methanol to carbon dioxide, so the result is an excessive consumption of methanol.

But some organisms have a different pathway, using glutamate dehydrogenase, which is less able to scavenge small amounts of ammonia but is more energy-efficient (and thus carbon-efficient). The possibility thus arose that the glutamate synthase gene in AS1 could be deleted, and the glutamate dehydrogenase gene substituted for it.

Luckily the well-studied *Escherichia coli* has the right gene, and Dr John Windass of ICI Corporate Laboratory was able to excise its gene and insert it into AS1. The greatest problem turned out to be how to isolate an AS1 mutant which had a deletion in the glutamate synthase gene: such mutants die immediately. However, ICI discovered a temperature dependent mutation — one where the glutamate synthase fails to operate above 37°C — and

Windass inserted the dehydrogenase gene into this mutant.

Above 37°C this modified strain uses the dehydrogenase pathway for assimilating ammonia; and over a two-months period the strain has proved to be stable.

Dr Windass, speaking at a recent biotechnology meeting in Rotterdam, claimed that the engineered strain produced a "significant" improvement in carbon efficiency. Questioned whether a 3 to 5% improvement would justify the long toxicological studies that would be required if the strain was eventually to be employed, another ICI representative, Dr Peter Senior remarked that it would.

Toxicology studies are underway on the new strain, but it would be "at least five years" before it could be used. The strain could be adopted in the newly commissioned SCP plant, but there its efficiency would be sub-optimal, said Senior. "You have to design your plant around your bug".

In fact, Senior believes that the future of biotechnology will not be all plain sailing. "All the easy things have been done", he went on. "Biotechnology is grossly overselling its potential, and there is little likelihood on both scientific and economic grounds that we are staring a revolution in the face".

Conceptually the simplest biotechnical process was the growing and harvesting of single cell organisms, said Senior, yet "from my experience the single cell protein processes that have been developed at ICI have stretched the imagination and innovative skills of all those involved in their development to a degree that the conventional chemical industry has not experienced before".

The ICI single cell protein process was first envisaged in 1968; in 1976 a sum of £40 million was sanctioned for the construction of a large plant, which is now on the point of production.

Robert Walgate

United Kingdom

No conclusion on low level lead hazards

A government report, published last week, has provoked strong criticism from the lobby demanding reductions in the amount of lead added to petrol. The report, *Lead and Health**, concludes that lead in air is not the most significant contributor to lead contamination in people: food and water are more important. "In the vast majority of the population", it says, "airborne lead, including that derived from petrol, is usually a minor contributor to the body burden".

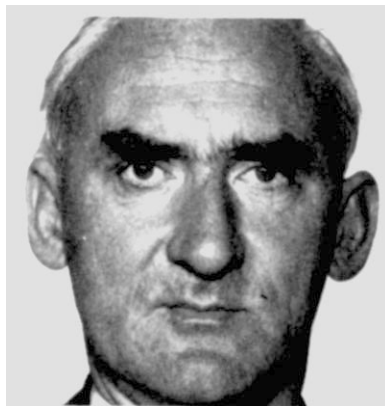
The working party on lead, set up in November 1979 under the Chairmanship of Professor P.J. Lawther, head of the MRC Toxicology Unit at St Bartholomew's Hospital, to prepare the report, concludes that it has only been able to satisfy one of the objectives required by its terms of reference — that of assessing the role of lead from petrol in relation to the other sources of lead in the environment. Its other aims — "to review the overall effects on health of environmental lead from all sources and, in particular, its effects on the health and development of children" — have been more difficult to achieve.

The toxicity of heavy exposure to lead is well known. The main controversy is over whether or not exposure to fairly low levels can effect health, especially the mental health of children. Several studies, in the US and Europe, suggest that children exposed to levels of lead which would not be high enough to result in the conventional signs of lead poisoning, have lower IQs and more behavioural problems than children who have not been exposed, or only very slightly.

The working party tried to meet the second part of its terms of reference by reviewing these studies. It concludes that there is no evidence of "deleterious effects at blood lead levels below about 35 $\mu\text{g}/\text{dl}$; neither is there any doubt about the serious consequence of blood lead levels above about 80 $\mu\text{g}/\text{dl}$; at intermediate concentrations, however, it says that the evidence is still very uncertain.

Many of the studies on the intelligence and behaviour of children are criticised in the report for not giving full details of their methodology. It says that in many of them, conflicting results have meant that the working party has not been able to come to any "clear conclusions concerning the effects of small amounts of lead on the intelligence, behaviour and performance of children". It recommends that further and more careful studies should be done: in particular better tests for examining intellectual function and behaviour in small groups of children are needed as is more research on the effects of pre-natal exposure on subsequent development.

Nevertheless, the report recommends



Professor P.J. Lawther, working party chairman

that children with blood lead levels above 35 $\mu\text{g}/\text{dl}$ should be followed up to identify the source of lead exposure.

Most cases of heavy contamination, the report concludes, are unlikely to result from exposure to unusually high concentrations of lead in air, although there will be local "hot spots" where airborne lead concentration are high and make a major contribution to overall body burden. Such areas should be identified and steps taken to minimise the hazard. The most likely sources of heavy exposure, however, are tap water, lead in paint and lead in imported cosmetics. The report recommends that a programme to detect lead in paint coatings accessible to young children be set up and that local authorities and water boards be encouraged to reduce lead in tap water in badly affected areas. The import and use of lead containing cosmetics should be discouraged, it says, and potential users should be made aware

of the hazard.

Other recommendations made by the report are the elimination of lead from manufactured foods — this is particularly a problem with canned foods — the development of guidelines for acceptable concentrations of lead in soil — more research also needs to be done on how plants take up lead from soil — and better education of the public on the hazards of lead especially to small children.

On the controversial topic of emissions of lead to the air from traffic, the report recommends that it should be progressively reduced and that the annual mean concentration of lead in air should be kept at less than 2 $\mu\text{g}/\text{m}^3$ in places where people spend a lot of time. Measures to achieve these limits may include "reduction of emissions, the relocation of industry or housing or traffic management schemes".

The report has been criticized by the anti-lead lobby. Parents Against Lead issued a statement at the time of the report's release saying that it had not taken into account evidence linking birth defects with lead contamination of the mother neither had it considered research already done showing that much of the lead in food comes from the air. To suggestions that the working party had been swayed by the oil industry, Professor Lawther said that each member of the party had their own scientific reputation to maintain and they had struggled to produce a fair scientific assessment of the subject.

Judy Redfearn

Lead and Health: the report of a DHSS Working Party on lead in the Environment HMSO £4.50

Dual support system for review

THE 'dual support' system whereby UK universities pay for buildings, services, technicians, basic laboratory equipment and salaries while research councils pay short-term grants for specific research is to be questioned by a new 7-man working party which will report ultimately to the Department of Education and Science.

The working party is "to review the current arrangements for the support of university research in the natural and social sciences". It is drawn from the Advisory Board for the Research Councils, which advises government on the allocation of the science budget among the research councils, and the University Grants Committee which provides universities with their basic running funds. The party will be chaired by Sir Alec Merrison, Chairman of the ABRC; other members include Dr Edward Parkes, Chairman of the UGC, and Sir Geoffrey Allen, Chairman of the Science Research Council (largest of the five research councils).

The feeling has been growing among the research councils that cut-backs in the UGC grant have fallen disproportionately on science, engineering, and medicine which through their laboratories have greater service requirements than the humanities. Applications to the research councils are increasingly including requests for equipment which would normally have been purchased through the university, and demands are increasing on research council central facilities (such as computers).

The working party will collect evidence, Sir Alec told *Nature* last week "and we can't rely on just a few scraps of information". On the other hand, the problem was too urgent to wait for the drafting and processing of a large questionnaire. The party "will not look at the total level of academic manpower" but will consider the problem of post-doctoral fellows and the provision of technicians.

"There is broad agreement that something is going wrong" with dual support

said Sir Alec. "We will have to balance saying something quickly with saying something reliable." The party hopes to report by the end of the year.

Meanwhile budgets for the research councils and the universities have fallen in real terms since 1978-9. Last week the UGC grant for the academic year 1980-1 was announced at £987 million, which says the Department of Education and Science "taking into account . . . the withdrawal of the subsidy for new overseas students . . . is about 2% lower in real terms than in 1979-80". The ABRC budget was also announced at £383 million for 1980-81 (see *Nature*, 3 April, page 000) with small increases for subsequent years, but these values are lower than those projected by the previous Labour government.

Jobs are also under pressure from these cuts, leading to a rapidly increasing average age for research groups; so it is hoped in some quarters that the terms of reference of the Merrison party will allow it to consider people as well as equipment.

● **EEC students to pay less:** The demand that foreign students must pay full tuition fees at British universities has been relaxed

for members of European Community countries, according to a report in *The Times*. This will increase pressure from Commonwealth countries (which account for half the UK's foreign students) that they also should get special treatment. The government is probably relying on the small number of EEC students in Britain remaining small, but, says *The Times*, it may have neglected the 3,500 Greek students who already outnumber EEC students. From 1981 Greece will be a member of the EEC and there may be an even greater inflow of students from the country.

● **£5 million for overseas student problem:** Mark Carlisle, Secretary of State for Education and Science, announced in the House of Commons last week that £5 million would be available in the academic year 1980-81 "to help ensure that uncertainty about prospective income from overseas students does not adversely affect selected postgraduate work of particular importance to this country". University sources last week had had no official indication of the meaning of this phrase.

Robert Walgate

Soviet Union

Academy stresses applied science

LAST November, Mr Brezhnev, in a major speech, charged the Soviet Academy of Sciences with greater responsibility for applied science and technology. Last month's annual general meeting of the Academy stressed that this new emphasis is well to the fore in its thinking.

How far this reflects a genuine change of direction is, of course, disputable. The Academy has already made it clear that it had been involved in applied research for some time before Brezhnev's speech. Nevertheless, the emphasis in this year's reports on the applied value of the Academy's work may be significant. President of the Academy Anatolii Aleksandrov, in his Presidential address, stressed particularly the work of the Shumyakin Institute of Bio-organic Chemistry, not only on protein structure, but also on genetic engineering, with, he implied, potential economic applications. He likewise commended the Academy's Institute of Microorganisms Biochemistry and Physiology for its work on artificial proteins for animal feed, based on oil products and natural gas, and in particular, the organic chemistry data bank established at Novosibirsk, which, he said, would enable scientists to develop new organic compounds with predetermined properties.

Physics and Geography Department of the Academy, Leonid Brekhovskikh outlined possible uses for the Soviet Union's glaciers and ice cover. His department, he said, had

devoted much time and resources to the study of glaciers as a fresh water source, since they contained "twice as much fresh water" as all the world's rivers and lakes. Ice could also, he said, be used as a building material in northern areas, when constructing port berths and laying roads.

From the newly exploited hinterland of the Baikul-Amur mainline railway, Academician Aleksandr Yanshin reported that after many years of forecasting and prospecting for potassium salts in Siberia, a major deposit, sufficient to ensure the "chemicalisation of agriculture in Siberia" had been located in the north of the Irkutsk oblast. While Academician Aleksandr Fokin, Deputy Chief Academic Secretary to the Presidium, in effect summarised the tone of the meeting by stating that great attention was being paid to links between research and production.

To carry out this massive programme, the meeting was told, Soviet funding for science had been raised by 55% during the past seven years (while the number of scientists actively involved in research had grown by 50% to 1,300,000).

In his Presidential address, Aleksandrov also stressed that, to solve such global problems as the search for new energy sources and the exploration of space, international cooperation was essential. He censured the US administration for curtailing Soviet-American scientific ties and exchanges.

Vera Rich

Soviet Union

Media accuses US of war-mongering

US allegations about a bacteriological warfare incident at Sverdlovsk were met by the Soviet Union not merely with protests that the US was attempting to wreck the Geneva review conference on bacteriological warfare, but also by counter-allegations that the US was preparing itself for chemical war. In a major media campaign, the Soviet Union has cited sources ranging from *Newsweek* to "documents of the Church of Scientology" to support claims that the US is amassing "wet-eye" (nerve gas) and "dry eye" (stable toxic agent) bombs, carrying out experiments with toxic chemicals on US citizens, building up twelve arsenals of nerve gas, bacteriological aerosols, narcotics, defoliants and herbicides, while working on a "new generation" of binary gas chemical weapon "causing death in a matter of split seconds".

Much of the radio material, in both Russian and English, was beamed at the Third World. Special emphasis was given to alleged CIA experiments on "coloured people" in order to test the vulnerability of different ethnic groups. And a whooping cough epidemic of the mid-1960s in Florida, which resulted in 12 deaths, was specifically attributed to the CIA.

Nor was Britain exempt from such charges — it was attacked for alleged work on bubonic plague virus (citing the *Daily Telegraph*, training in the use of toxins at the army staff college (a "recent British TV film") and the new firing range near Porton Down (*Now!*), "officially, being used to devise protection against chemical warfare").

A few days after Tass's accusation, in the "Russian for abroad" service of Moscow radio, that the US are working, in "top-secret military laboratories", to "cultivate bacteria which could cause mass epidemics like anthrax, typhoid, plague, smallpox, etc", it had to elaborate on the official explanation of the Sverdlovsk incident. It said it was an outbreak of anthrax, caused by "adverse weather conditions in the autumn-winter of 1978-79" (which made sheep and cattle susceptible to contagious diseases), lack of personal hygiene in tending livestock, and the purchase of unbranded meat, wool and hides from unauthorized individuals. In spite of the struggle against the disease, it was explained, anthrax has never been completely eradicated from the Urals — thereby unfortunately providing a weapon for the US hawks to "call into validity [the bacteriological weapons] convention" and to "whip up the arms race".

Vera Rich

NEWS IN BRIEF

US produces draft climate plan

RESEARCH into the impact on the environment and society of increased carbon dioxide levels in the atmosphere, and into the effects of climatic change on world food production, are two of the top priorities that have been selected for attention under the draft of a National Climate Plan, published in Washington last week.

The plan presents a five-year programme for climate research, as required by the National Climate Programme Act of 1978. The carbon dioxide study has been selected as a "principal thrust" by the Department of Energy and the Department of Agriculture.

Also under the proposed climate plan, the National Aeronautics and Space Administration will give priority to efforts at understanding how the climate system gains and loses radiant energy. And the National Science Foundation will lead a major coordinated effort to understand the oceans' role in climate, in particular with heat-flux experiments planned in the Atlantic Ocean, perhaps leading to "a series of major international experiments in the late 1980s and early 1990s". The climate programme will coordinate the research efforts of all federal agencies, with the National Oceanic and Atmospheric Administration (NOAA) acting as the lead agency. NOAA's principal thrusts will be in the generation and dissemination of climate information and climate prediction.

US agency denies Agent Orange cover up

OFFICIALS of the Veterans Administration denied congressional charges last week that an official of the administration had referred to the herbicide 2,4,5-T as carcinogenic and mutagenic in a memorandum written in 1977. The charge had been made by two congressmen last month, who had obtained a copy of a memorandum with 'US government' written across the top, which said that the herbicide, known as Agent Orange, had been shown to "intercept the genetic DNA message process to an unborn fetus, thereby resulting in deformed children being born".

In producing the memorandum, the congressmen accused the administration of giving misleading information when it had earlier testified that there was no evidence linking the herbicide with human health problems. However, in a reply, Mr Max Cleland, administrator of the Veterans Administration, said that the VA official, whose telephone conversation was supposed to be the source of the memoran-

dum, had pointed out that it was "inaccurate in numerous points". Mr Cleland said that no-one in the VA had seen the memo before, and that it had been impossible to identify the author.

Australia orders Agent Orange inquiry

THE Australian government announced last week its intention to study the effects of Agent Orange, the dioxin containing herbicide used by the US to defoliate the countryside in Vietnam. Following a campaign by community and servicemen's groups, the government has granted \$2 million to survey the health of 41,000 Australian servicemen who were exposed to the chemical during the Vietnam war. In addition, the health of the families of the exposed servicemen will be studied. The study will be carried out by the Commonwealth Institute of Health of Sydney University. Veterans organisations have criticised the proposal saying that the survey will take too long to complete to prevent injury to those exposed. Mr K.G. Schultz, national secretary of the Returned Services League, said that a smaller sample would provide sufficient information about ill effects faster than the full survey. The Vietnam Veterans Action Association said it already had an overwhelming case for compensation with cases of birth defects that included missing limbs, club feet and cleft palates.

UK minister welcomes ASTMS cancer document

THE UK Secretary of State for Employment stated in parliament that he welcomed the Association of Scientific, Technical and Managerial Staffs document on occupational carcinogens (*Nature*, 20 March, page 203). In a written response to a parliamentary question by Renee Short asking if the government will take action "as a matter of urgency" on the document the minister said: "I welcome the initiative this union has taken in preparing the document, which I understand is to provide safety representatives and union officers with information. I propose to await advice from the chairman of the Health and Safety Executive before deciding whether discussions with the HSE or other interested parties are necessary".

UKAEA agrees PWR cracks a hazard

In a supplementary memorandum to the select committee on energy, the UK Atomic Energy Authority has agreed that the possibility of pressure vessel cracks

constitutes an insufficiently explored hazard of pressurised water reactors according to a report in *The Times*. The memorandum agrees with the analysis of Sir Alan Cottrell (*Nature*, 28 February page 803) that cracks as long as one inch could develop without detection in the pressure vessel which could lead to a rapid uncontrollable break resulting in a complete loss of coolant accident. Development of more advanced detection techniques than were previously thought necessary is required before the American designed PWRs can be used in the UK. "It is vital that the necessary development work be undertaken as a matter of urgency" the memorandum is reported as saying.

In the meantime, Britain's ten year plan to introduce PWRs starting in 1982 may be delayed for another reason. The Nuclear Installations Inspectorate told the select committee last week that the Central Electricity Generating Board had yet to present its PWR design for safety clearance and to conclude a licensing agreement with Westinghouse. The delay, senior NII officials said, meant that the necessary safety studies could not be completed by the 1982 deadline.

In related developments, the Prime Minister is withholding approval for the construction of two scheduled advanced gas cooled reactors. The Prime Minister is a strong advocate of the PWR and has ordered further studies of the AGR proposal.

● Mr David Steel, leader of the Liberal Party, criticised Mrs Thatcher's "nuclear obsession" at the UK's largest ever anti-nuclear rally last week held on the anniversary of the Harrisburg accident. Fifteen thousand demonstrators from many British anti-nuclear groups attended.

Debendox safe, says UK committee

THE UK committee on the Safety of Medicines has found no evidence that the drug Debendox, an anti-nausea drug used by pregnant women, causes birth defects. The committee completed its third review of the drug at the end of last month. Two previous reviews in 1978 and 1979 had also found that the drug was safe.

Concern about Debendox has been stimulated by the recent court case in the US — where the drug is manufactured under the name Bendectin — which found that it had caused congenital abnormalities in a boy. The UK Minister of Health asked the committee to review its safety.

The committee has said that the level of abnormalities in the children of the 3,500,000 women who have taken the drug in the UK over the past 20 years, is no more than in the population as a whole.

NEWS AND VIEWS

Is the Sun still shining?

from F.E. Close

THE conjunction of cosmology and particle physics as the result of recent developments in high energy physics formed the subject of a Wolfson Invitation Lecture given at the University of Oxford by J. Ellis of CERN. Taking as his title 'The very large and the very small' one of his themes was the current interest in neutrinos and how an understanding of their physics can provide explanations for some apparent anomalies in the Sun's interior.

Neutrinos are ghostly particles that carry no electrical charge and interact visibly only by means of the weak force. They are produced in stars, like the Sun, when light nuclei fuse to form heavy nuclei with consequent release of energy. They stream out from the Sun and can be detected on Earth by monitoring their reactions with atomic nuclei. In particular, their reaction with chlorine, which is readily available in ordinary cleaning fluids is used to calculate the incident neutrino flux.

When neutrinos interact with the ^{37}Cl nuclei in large underground tanks of cleaning fluid they initiate the reaction



by a neutron-proton conversion. The argon produced is extracted by helium purging, separated from the helium mass and finally introduced into a small proportional counter. Argon-37 undergoes K-capture decay with a 35 day period. Almost all these K capture events are detected and so the amount of argon is inferred and in turn the incident neutrino flux responsible for creating it in the first place can be calculated. This quantity is expressed in solar neutrino units (SNU). But although theoretical understanding of the Sun's interior requires that about 6 ± 2

SNU be observed, experiment has detected only a fraction of this. (A meeting in Rome during early February gave 2.2 ± 0.3 as the most up-to-date result).

One possibility is that our theoretical understanding of the Sun is badly wrong somehow. The next source of blame is in the inferences drawn from the experimental rate of detection.

Detection exploiting chlorine nuclei has several attractive features, not least of which is the abundant availability of cleaning fluid. However, a great disadvantage is that the transition can only be triggered by neutrinos that have at least 0.8 MeV energy. This is far above the maximum energy of neutrinos produced in $p + p \rightarrow d + e^- + \nu$ which is the main thermonuclear reaction in the Sun, and so the detection technique is insensitive to this important flux of neutrinos.

These low-energy neutrinos could be picked up through the nuclear transition from gallium-71 to germanium-71, which can be initiated by neutrinos with energies as low as 0.23 MeV. But to gain such information a target containing several tons of gallium would be required which is larger than the annual world production. Even were this region of the spectrum better studied however, it is thought unlikely that all the discrepancy with theory would be removed.

Another possibility is that the Sun has stopped shining. If the thermonuclear processes in the heart of the Sun have run down, there will be a significant drop in the neutrinos produced and a low flux will be detected on Earth. This is because the Sun is almost transparent to neutrinos and so they stream out from the core unhindered. In contrast, the Sun is quite opaque to photons and those that irradiate the Earth have come from the Sun's outer reaches. It would be some hundreds of thousands of

years before news of the changed circumstances in the Sun's core reached its surface. Thus we could be somewhere in this period, waiting for the surface photons to be turned off — the low neutrino flux is our advance warning of a real energy crisis.

Fortunately there is another possible cause for the low flux which is of particular interest in light of recent discoveries in particle physics.

Before 1976 two varieties of neutrino were known — one related to the electron (called electron-neutrino or ν_e), and one partnering the muon (muon-neutrino, ν_μ). Their masses are known to be orders of magnitude smaller than the lightest known particle (electron) and so it has been supposed that the neutrinos are massless. Since 1976 a third variety of neutrino has been discovered associated with the heavy lepton tau (τ). This ν_τ is less well studied but is also often referred to as 'massless'.

Masslessness and conservation principles are intimately linked, for example, the conservation of electrical charge is related to the masslessness of the photon. There is no conservation principle known that would require the neutrinos to be massless and so the possible consequences of their having masses have been studied.

If neutrinos have masses then the mass eigenstates could be linear superpositions of the ν_e, ν_μ, ν_τ eigenstates. Thus an electron-neutrino emitted in the Sun would be a linear superposition of three different mass eigenstates, each of which will oscillate with slightly different frequencies during the journey across space. By arrival at Earth a different linear superposition of mass eigenstates will be present which is equivalent to saying that what started out as ν_e is now a mixture of ν_e, ν_μ and ν_τ . If the mass differences are a few electron volts then the oscillations could be sufficiently rapid that the intensity of ν_e

arriving at Earth could be as low as 1/3 that which set out (the 1/3 being due to the three possibilities ν_e, ν_μ, ν_τ). This would naturally fit with the observed solar neutrino flux which is about 1/3 of that expected in the absence of oscillations.

The solar neutrinos produced in radioactive decays have a low energy. Higher energy neutrinos such as those produced in particle accelerators will have correspondingly larger oscillation lengths. As neutrinos can pass through the Earth without attenuation one could put detectors in line with a neutrino beam at CERN and see if the number of ν_μ , say, varies with distance.

One interesting theoretical development is the idea that all nature's forces could be unified at very high energies. Some versions of these theories predict that neutrinos have masses inversely propor-

tional to the unification scale. The unification is believed to occur at the order of the Planck mass (10^{19} GeV). This is large but not infinite and so the neutrino masses are small but not zero, of order 10^{-5} electron volts. If neutrino masses are indeed of this order then oscillation effects could be directly observable. In particular, the eccentricity of the Earth's orbit gives a natural variation to the distance that neutrinos have travelled *en route* from the Sun. Neutrino masses of the order of 10^{-5} eV could account for the solar neutrino anomaly, and provide an annual periodic variation in intensity of electron neutrinos which could be looked for. Such an observation would be exciting in its own right, would indirectly support attempts to unify the natural forces and would indeed resolve the solar neutrino anomaly, avoiding the catastrophic alternative. □

Iron, plasmids and infection

from E. Griffiths, Henry J. Rogers & J.J. Bullen

AN important development in our understanding of bacterial infections has come from the realization that genetic determinants for certain virulence characteristics, such as haemolysins, toxins and adhesive factors, can be carried by plasmids. In this issue of *Nature* (page 566), J.H. Crosa reports on the presence of a plasmid in highly virulent strains of the fish pathogen *Vibrio anguillarum* which mediates a novel virulence mechanism, that of an efficient iron sequestering system. On losing the plasmid, *V. anguillarum* loses its virulence and also its ability to grow under conditions of iron restriction imposed by the serum iron binding protein, transferrin.

Several years ago it was found that iron compounds could abolish the antibacterial effects of body fluids *in vitro* and enhance bacterial virulence *in vivo*. These studies have led to an increased knowledge of the mechanisms of resistance to bacterial infection and have also provided an explanation for the changes in resistance which often accompany alterations in the iron status of the host (Bullen *et al. Curr. Top. Microbiol. Immun.* **80**, 1; 1978; Weinberg *Microbiol. Rev.* **42**, 45; 1978). We now know that although there is plenty of iron present in the body fluids of vertebrates, the iron content of human plasma for example is 20 μ M, the amount of free iron is of the order of 10^{-18} M, which is far too low for bacterial growth. This extremely low concentration of free

iron is due to the presence of iron binding proteins, transferrin in blood and lymph and lactoferrin in secretions, with which the ferric ion is very strongly associated. Pathogenic organisms which multiply successfully under these conditions must possess mechanisms for assimilating protein bound iron or acquiring it from liberated haem.

Several organisms are known to produce iron chelating agents. *Vibrio cholerae*, for example, produces a phenolate iron chelator (Payne & Finkelstein *Infect. Immun.* **20**, 310; 1978) and *Pseudomonas aeruginosa*, iron chelators of both the phenolate and hydroxamate type (Liu & Shokrani *Infect. Immun.* **22**, 878; 1978). The best studied system, however, is that in bacteria of the genera *Salmonella*, *Escherichia* and *Klebsiella* which secrete the iron chelating compound enterochelin under iron restriction *in vitro*. This compound, which is the cyclic trimer of 2,3,4-dihydroxybenzoyl serine, removes iron from iron-binding proteins and it has been proposed that its secretion is essential for growth of the organisms *in vivo* (Rogers *Infect. Immun.* **7**, 445; 1973). Recently, Griffiths and Humphreys (*Infect. Immun.*, in the press) have shown that enterochelin is produced *in vivo* during fatal infections with *E. coli*. Yancey *et al* (*Infect. Immun.* **24**, 174; 1979) have reported that the loss by mutation of the ability to synthesize enterochelin greatly reduces the virulence of *Salmonella typhimurium* and also inhibits its ability to grow in human serum. In addition to secreting enterochelin, *E. coli* growing under iron-restricted

conditions produce new outer membrane proteins which act as receptors for Fe^{3+} -enterochelin (McIntosh & Earhart *Biochem.* **81**, 749; 1977). These organisms also contain altered species of tRNAs (Griffiths & Humphreys *Eur. J. Biochem.* **82**, 503; 1978). The same tRNA changes are found in *E. coli* recovered from the peritoneal cavities of lethally infected animals (Griffiths *et al. Infect. Immun.* **22**, 312; 1978). The alterations, which arise from an undermodification of the hypermodified nucleoside 2-methylthio- N^6 -(Δ^2 isopentenyl) adenosine in the tRNAs during growth under iron restricted conditions, may play an important part in regulating the expression of operons of the aromatic amino acid biosynthetic pathway from which enterochelin is synthesized by way of a branch pathway (Griffiths & Buck *Soc. Gen. Microbiol. Quart.* **7**, 10; 1979).

In a recent issue of *Infection and Immunity* (**26**, 925; 1979) P. Williams shows that *E. coli* carrying colicin V (Col V) plasmids possess another iron-sequestering mechanism, in addition to the enterochelin system, and suggests that this may be a contributory factor in the virulence of invasive strains. A well established link exists between Col V plasmids and the invasiveness of pathogenic *E. coli*. Most *E. coli* strains isolated from cases of bacteraemia in humans and domestic animals harbour Col V plasmids. Although the possession of a Col V plasmid markedly enhances virulence, colicin V activity itself is not essential (Binns *et al. Nature* **279**, 778; 1979; Quakenbush & Falkow *Infect. Immun.* **24**, 562; 1979). Williams finds that the possession of the Col V plasmid usually allows the bacteria to grow significantly faster in the presence of transferrin. However, this is not always the case. One Col V plasmid, which did not confer any growth advantage in conditions of iron stress, has been shown by Binns *et al.* to carry determinants for resistance to the bactericidal effects of serum. The acquisition of either characteristic, serum resistance or an enhanced ability to grow under iron stress, could confer a selective advantage on *E. coli in vivo*. The acquisition of both determinants would give an organism an even better advantage. It remains to be seen whether or not the plasmid in *V. anguillarum* mediates other virulence determinants in addition to enhancing the ability of the bacteria to grow in iron-restricted conditions.

The presence of a plasmid is not, of course, always required for virulence, nor is it the only factor involved. A further complication in the case of Gram negative pathogens is the natural variation in the O and K antigens of the bacterial cell wall. There is undoubtedly a connection between the structure of these complex polysaccharides, which determine serological specificity, and the virulence of the organism (Orskov *et al. Bact. Rev.* **41**, 667, 1977; Merson *et al. Infect. Immun.* **23**, 325;

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1979). There are *E. coli* strains for example which belong to classically recognized enteropathogenic serotypes that are non-toxicogenic and non-invasive according to the usual criteria, and do not seem to harbour virulence plasmids but which nevertheless do cause severe disease (Levine *et al. Lancet* **1**, 1119; 1978; Scotland *et al. FEMS Microbiol. Lett.* **7**, 15; 1980; Ulshen & Rollo *New Engl. J. Med.* **302**, 99; 1980). In an era when resistance to known antibiotics seems to be increasing we clearly need to know far more about the mechanisms of microbial pathogenicity and host resistance. Although good progress is now being made, it is more than likely that the versatile bacterium still has many more surprises in store for us. □

X-ray astronomy with the Einstein observatory

from J.L. Culhane

NASA's Einstein X-ray observatory satellite was launched in November 1978. It carried a large reflecting X-ray telescope, of a few arc seconds angular resolution, equipped with various focal plane detectors for X-ray imaging and spectroscopy. Before its launching, X-ray astronomy had made spectacular advances through observations undertaken with satellites instrumented by US, UK and Netherlands research groups. These missions established the existence of several hundred X-ray sources and discovered a range of exciting phenomena that included the presence of rotating neutron stars and perhaps even black holes in binary star systems, the existence of high temperature gas spread throughout clusters of galaxies, the identification of Seyfert galaxies as X-ray sources and the heating of interstellar plasma to million degree temperatures by the shock waves that expand from supernova explosions. However with the exception of the small X-ray reflectors on the Copernicus satellite, and the modulation collimators on SAS-3, Ariel V and HEAO-1, all of these missions used collimated proportional counter detectors with fields of view of several square degrees. Thus it was to be expected that the availability of a truly imaging telescope with arc second resolution would bring about a transformation in our knowledge of the X-ray sky.

A preliminary measure of this transformation is provided in a recent issue of *Astrophysical Journal Letters* (234, L1-L81; 1979) which contains 15 papers devoted to the first results obtained with

the Einstein satellite. The observing programme included many specific objects but, because of the two order of magnitude improvement in sensitivity over previous instruments, it was also decided to use the telescope for deep surveys of several regions of the sky that contained no previously known X-ray sources. Regions in Draco and in Eridanus have been studied and 43 new sources identified. Ten of these are believed to be extragalactic, the majority probably quasars and on the basis of an extrapolation to lower fluxes, the contribution of such sources to the diffuse X-ray background is established as almost 40%. If the majority of these sources were indeed quasars and active galaxies, then on the basis of our current understanding of X-ray to optical luminosity ratios, quasars brighter than $B \sim 20.3$ mag could account for essentially all the X-ray background. If confirmed by future observations, this could prove to be one of the most significant discoveries made by Einstein. Quasars are in fact established as a class of luminous X-ray emitters by the detection of more than 30 where only 3 X-ray quasars were known before the Einstein mission. Many more are being found as observations continue.

It has been known for some time that the giant radio galaxy, Centaurus A, has an X-ray source located in the nucleus of the associated active galaxy. Observations with the Einstein telescopes have now established that in addition to the point X-ray source coincident with the infrared nucleus, a diffuse component associated with the inner radio lobes, an emitting region of about 4 arc min extent surrounding the nucleus and an X-ray jet lying between the nucleus and the NE inner radio lobe are also present. The paper which reports these discoveries suggests that the radio lobe component may arise from the inverse Compton scattering of the microwave background while the existence of a jet provides further evidence of the continuing supply of energy to the lobes from the nucleus. This observation of X-ray structure around the nucleus of the best studied giant radio galaxy could be of the utmost importance in helping us understand the energy supply mechanism for radio galaxies in general.

The extended sources of X-ray emission associated with clusters of galaxies have naturally been objects of much interest to the Einstein observers. Several papers are devoted to results on the structure and evolution of these sources. However one of the most striking observations has been the detection, from the neighbourhood of M87 in the Virgo cluster, of an emission line characteristic of ionized oxygen (hydrogen-like O^{+7}) by the focal plane crystal spectrometer. The detection of highly ionized iron (Fe^{+24}) emission lines by the Ariel V and OSO-8 satellites in the X-ray spectra of several clusters had previously established the presence of high temperature ($30 \times 10^6 \text{ K} < T < 120 \times 10^6$

K) plasma in these sources. However the detection of material around individual galaxies at temperatures less than 10^7 K has important implications for theories of gas infall and cooling in clusters. More recent observations with both the crystal and solid state spectrometers have indicated a similar situation near NGC 1275 in the Perseus cluster and we await further observations of the gas near massive galaxies in other clusters with considerable interest.

The Uhuru and Ariel V surveys indicated the presence of many X-ray sources in the plane of our galaxy thus suggesting that similar objects should exist in other normal galaxies. A dramatic measure of the advance due to Einstein is provided by the detection of 69 discrete and 7 diffuse sources in the nearby spiral galaxy M31. This is all the more striking when we recall that the Uhuru catalogue listed M31 as a single X-ray source with an intensity that was only just detectable. Thus the enormous power of a high resolution imaging telescope is clearly demonstrated. Similar advances have been made in observations within the galaxy. A group of six point sources has been found in the neighbourhood of Cygnus X-3 where previously only the latter was known to exist while the existence of more than 20 sources has been established in the Orion nebula. Work in progress at the Harvard Centre for Astrophysics which has been reported at a recent conference in Boston although not presented in the Einstein issue of *Astrophysical Journal*, suggests that X-ray emission has been detected from essentially all of the star classes represented in the H-R diagram. Thus X-ray astronomy has in less than two decades advanced from a concern with a small number of unusually energetic objects to occupy a position of central relevance for the entire field of astrophysics.

While the discovery of X-ray emission from a large number of stars is of considerable importance, a hint of the real potential of X-ray astronomy is provided by the beautiful emission line spectrum of the Capella system that has been obtained with the solid state spectrometer. X-ray emission lines from Mg, Si, S and Fe are observed and it is at once clear that the deployment of high resolution X-ray spectrometers will allow us to apply the sophisticated plasma diagnostic techniques, that have been refined over many years in observations of the solar corona and of laboratory plasmas, to the study of the detailed physics of a wide range of objects. The importance of X-ray spectroscopy is further emphasized by the X-ray emission line spectrum of the supernova remnant Cassiopeia A, which when compared with the detailed map of the remnant obtained with the imaging detectors, permits a considerable increase in our understanding of these objects.

Although the Einstein observatory has carried out a series of truly staggering observations in its first year of operation,

one cannot help but feel a slight disappointment that as yet nothing has emerged to rival in excitement the discovery of X-ray emitting binary systems and clusters of galaxies by the Uhuru satellite. So far no faint new categories of X-ray objects have been discovered. However to complain is merely to emphasize the extraordinarily high standards that have been set in the subject from the beginning. We are witnessing the establishment of a mature astronomical discipline which, along with optical and radio astronomy, must from now on have an essential role in our study of the Universe. The manner in which the Einstein mission is bringing about this maturity will surely come to be regarded as one of the substantial achievements of twentieth century astronomy. □

Exploiting papyrus

from Peter D. Moore

THE potential of papyrus (*Cyperus papyrus*) to 'clean up' tropical lakes in danger of eutrophication (see *News & Views* 276, 560; 1978) is again under review. In particular work by Gaudet (University of Nairobi) on Lake Naivasha in Kenya provides grounds for optimism.

Papyrus forms floating swamps in the peripheral zones of tropical lakes especially where incoming rivers debouch their waters (Gaudet *Aquatic Bot.* 3, 1; 1977). The plant is robust (up to 5 m tall) and productive, so considerable quantities of nutrient ions are absorbed during swamp growth. Gaudet (*Ecology* 58, 415; 1977) made some preliminary estimates of nutrient uptake based on certain fairly simple assumptions concerning the rates of culm replacement within a papyrus stand. He concluded that rates of uptake could amount to N, 62 kg m⁻²; P, 5 kg m⁻²; K, 103 kg m⁻² during one complete turnover of culm replacement (147 days). Nutrient losses would occur at the same time as a result of death, decay and leaching of older culms. Losses were considered to amount to two thirds of the material absorbed, the remainder accumulating within the swamp peat which descends from the floating mat to accrete with the other sediments below.

When inflow is high, water will move faster below the floating raft and will dilute the ionic concentration in this zone. On the other hand, in dry periods the concentration beneath the raft will rise as a result of evaporation. Fast through-flow could, of course, reduce the efficiency of the swamp in modifying water quality. Gaudet has monitored these effects through the year at Lake Naivasha (*Verh. Int. Verien Limnol.* 20, 2202; 1978) and has indeed shown such seasonal variations.

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The overall nutrient trapping efficiency of the swamp, however, was low as a result of the rapid water movement in wet periods. The final retention of nutrients in the sediments below the mat was of the order of 10% for magnesium, ranging down to 1% for calcium. So overall, the swamp is not greatly influencing water quality.

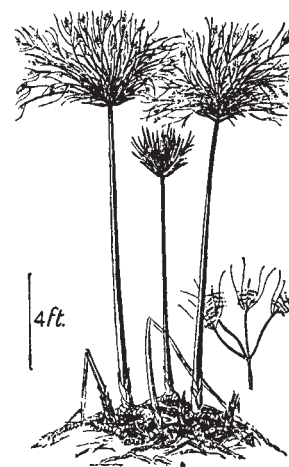
These figures may not fully reflect the precise operation of the process, however, because of the particular analytical techniques used. All water samples were filtered before chemical analysis, which would have removed organic particulate matter. Gaudet has now published additional data (*J. Ecol.* 67, 953; 1979) based on the use of sediment traps, and has expressed these as a percentage of annual input retained in the sediment. On this basis, the removal of elements is of the order P, 57%, S, 59%; Fe, 83%, Mn, 94%. So the swamp is acting as an efficient nutrient extractor, but much of the extracted material is being transported as detritus by the flow-through below the mat.

Nitrogen is an important exception, for there is a high degree of net fixation within the swamp system. Net gains within the ecosystem suggest fixation rates in excess of 50 g m⁻² annum⁻¹.

The efficiency of papyrus swamps as nutrient extractors would be increased if they were harvested periodically and the biomass removed. Thompson (in *The Nile, biology of an ancient river* (ed. J. Rzoska) 177, Junk, The Hague, 1976) has found that a stand of papyrus contains a mixed population of culms of various ages, with the life cycle of a culm taking about 150 days. Gaudet has shown that rapid accumulation of nutrients occurs in the early stages of growth; thus frequent cropping should result in a maximum nutrient drain on the system.

Thompson, Shewry and Woolhouse (*Bot. J. Linn. Soc. Lon.* 38, 299; 1979) have now conducted a detailed analysis of the population structure and productivity of papyrus culms in swamps of the Upemba basin of Zaire and in Uganda. The total biomass of the system seemed to be climatically determined, being 2.9 kg m⁻² in Uganda (545 m altitude), 1.1 kg m⁻² in Zaire (~1000 m) and 3.4 kg m⁻² at Gaudet's Lake Naivasha, Kenya (1830 m). But there was not such a strong, nor such a consistent variation in productivity between these areas; figures ranged between 10 and 35 g m⁻² day⁻¹. Some temperate reedswamps, such as *Scirpus lacustris* in Germany (*Westlake Biol. Rev.* 38, 385; 1963) have been described which overlap the lower end of this range, but these figures do emphasize the high productivity of *Cyperus papyrus*, which is considered by Thompson *et al.* to be a C₄ species.

So papyrus harvesting could provide a renewable energy resource for combustion at the same time as enhancing its efficiency in recovering nutrients from eutrophicated



Cyperus papyrus

waters. Work of the sort described here emphasizes the need to conserve tropical swamps until their full value can be assessed and until appropriate management regimes can be developed on the basis of controlled experimentation. Their value for local human populations could be considerable and their exploitation could relieve pressure on other, less easily renewed, ecosystems. □

Atomic hydrogen stabilized

from P. V. E. McClintock

SILVERA and Walraven's report (*Phys. Rev. Lett.* 44, 164; 1980), that they have succeeded in stabilizing bulk atomic hydrogen, suggests that the preparation of superfluid hydrogen may become a practical proposition in the not too distant future.

Superfluids, possessing the remarkable facility of frictionless flow behaviour, devoid of the viscous effects which characterize familiar liquids such as water, are exceedingly rare. The only examples known at the moment are the liquid heliums (⁴He below 2 K; ³He below 2 mK) and, in a slightly different sense, the electron gas in a superconducting metal. Thus if hydrogen and also, perhaps, its isotopes deuterium and tritium could be prepared in a similar state it would, at a stroke, double the number of superfluids accessible to experimental investigation.

This exciting possibility rests on the fact that, although ordinary molecular hydrogen (H₂) forms a solid at low temperatures, and is thus unable to become superfluid, no such restriction applies in the case of atomic hydrogen (H). The forces between a pair of hydrogen atoms depend profoundly on whether their electron spins lie mutually parallel or

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antiparallel. In the latter case, there is a very strong force of attraction, rapidly leading to formation of an H_2 molecule. In the former case, on the other hand, the interatomic force is so weak that it is possible, at least in principle, to cool a dense assembly of H atoms to the very low temperatures where superfluidity may be expected to manifest itself, without solidification occurring at all. The main difficulty, of course, is that of getting atomic hydrogen into a stable spin-aligned state, given that it has a very strong natural inclination to minimize its energy by forming H_2 .

Although Silvera and Walraven have followed the conventional approach of attempting to stabilize the H gas by a combination of high magnetic field and low temperature, their apparatus incorporates a number of novel and imaginative features. The atomic hydrogen itself was produced in the usual way by means of a discharge tube at room temperature but, rather than allowing the H gas merely to diffuse into the stabilization cell, they pumped it in using a specially designed vapour diffusion pump which, using helium as the working medium, possessed a unique capability in being able to operate at 0.48 K. This device was an essential feature of the overall experimental design because, as the same authors demonstrate in the paper immediately following (Walraven & Silvera *Phys. Rev. Lett.* **44**, 168; 1980), the H gas would otherwise have had a strong tendency to leak out of the stabilization cell again.

The authors also found that it was essential to cover the walls of the cell with a very thin layer of helium-4. Because hydrogen is insoluble in liquid helium, this coating served to insulate the H atoms from contact with the walls which, if it had occurred, would have produced spin-flips rapidly followed by recombination to ordinary molecular hydrogen.

A special type of bolometer was developed to demonstrate the presence of the H gas confined within the cell. This exploited the heat of recombination which is generated when H_2 is formed: after a controlled burning off of its helium coating, the bolometer provided an active surface on which recombination could take place. An estimate of the initial H density could be made by noting the extent of the recombination heating which occurred when the bolometer was triggered in this way. It was found that, after being filled, the cell could be left a period of several minutes without any measurable loss of H at all, implying that, at least within the timescale of the experiment, their stabilization scheme had been completely successful. The greatest density of H achieved was estimated as being not less than 1.8×10^{14} atoms cm^{-3} ; but the authors hope to be able to improve on this figure by several orders of magnitude (a factor of 10^5 or 10^6 being needed before the superfluid

effects would be expected), once they start trying.

These experiments represent a most encouraging step forward. They have raised the status of superfluid hydrogen from little more than a scientific pipe dream to being a very real possibility within the next few years. □

Are abundant proteins less variable?

from Yvonne Edwards & David A. Hopkinson

THE analysis of enzymes using gel electrophoresis and specific activity stains has revealed extensive genetic diversity in man and other species. At least 25% of the genes which determine enzyme structure are variable and the average level of heterozygosity per gene locus detectable by conventional unidimensional electrophoresis is in the region of 7 to 10% in man and other vertebrates and somewhat higher in insects such as *Drosophila* (Harris *et al. Ann. Hum. Genet., Lond.* **36**, 9; 1972). However, recent studies on the same species using the O'Farrell bidimensional gel electrophoresis technique (*J. biol. Chem.* **259**, 4007; 1975) suggest that the average heterozygosity at gene loci determining the particular array of proteins discernible by this procedure is about 1 to 4%, substantially lower than expected from the enzyme surveys. Are the data from the uni and bidimensional electrophoresis compatible?

The bidimensional technique provides a method for the analysis of proteins which occur in greater abundance than most of the enzyme proteins since it relies on non-specific protein staining or autoradiography. The proteins are denatured and separated by isoelectric focusing in the first dimension according to charge and by SDS gel electrophoresis in the second dimension according to molecular size. A large number of gene products can be analyzed in a single experiment, but side-by-side comparison of the complex patterns of several hundred spots is difficult and the identification of individual variation is not as straightforward as in the unidimensional electrophoresis of enzymes. However, two recent studies (McConkey *et al. Proc. natn. Acad. Sci. U.S.A.* **76**, 6500; 1979; Walton *et al. J. biol. Chem.* **254**, 7951; 1979) on the polypeptides of human fibroblasts are noteworthy since they elegantly solved the problem of side-by-side comparison by using a double label autoradiographic technique which permits

the simultaneous analysis of 3H -labelled and ^{14}C -labelled proteins in the same gel. Between 300 and 400 polypeptide spots from human fibroblasts were examined and the average heterozygosity was found to be in the region of 1%. Estimates of average heterozygosity lower than expected from enzyme surveys were also found in bidimensional electrophoretic analyses of smaller samples of *Drosophila* (Leigh Brown *et al. Proc. natn. Acad. Sci. U.S.A.* **76**, 2381; 1979) and mouse polypeptides (Racine *et al. Nature* **283**, 855; 1980).

The reasons for the apparent discrepancy between the results obtained by the different techniques are not clear, but there are two major lines of argument worth exploring. Either the polypeptides accessible to analysis by the bidimensional procedure derive from a class of proteins with genetic properties distinct from those of soluble enzymes or this method is not as efficient at detecting variant proteins as conventional electrophoresis.

Bidimensional analysis selects those polypeptides present at highest concentrations, but this feature almost certainly does not account for the apparent low incidences of genetic variation. On the contrary, there is good evidence that the relative abundance of a protein and levels of heterozygosity at particular gene loci are not directly related, for example amongst human proteins present at high concentration haemoglobin, carbonic anhydrase II and several of the serum proteins show genetic polymorphism. And more recently, extensive genetic heterogeneity of the major storage protein of *Pisum*, demonstrated by both uni and bidimensional analysis, has been reported (Casey *Heredity* **43**, 265; 1980).

However, the array of proteins analyzed by the two procedures differ not only in their relative abundance, but also in their subcellular origin. In the bidimensional system whole cells, tissues or organisms are solubilized by denaturation, so that many of the most abundant proteins are derived from structural components such as membranes and are very likely to have complex subunit structures. It can be argued that vital protein-protein interactions and associations between protein and lipid/carbohydrate moieties such as occur in membranes, place constraint on the number of mutations which can occur. Furthermore, it is now recognized that multimeric proteins involved in polypeptide interactions show less genetic variation than monomeric proteins (Harris *et al. Proc. natn. Acad. Sci. U.S.A.* **74**, 698; 1977; Zouras *Nature* **262**, 227; 1976; Ward *Biochem. Genet.* **15**, 123; 1977) and it is possible that multimers may be over-represented in the array of proteins analyzed by the bidimensional procedure.

Considering the question of efficiency there is a considerable body of evidence which testifies to the discriminatory power

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of the bidimensional method. Milman *et al.* *Proc. natn. Acad. Sci. U.S.A.* **23**, 4589; 1976) detected a mutation for the enzyme hypoxanthine guanine phosphoribosyl transferase by analysis of HeLa cell extracts, Commings (*Nature* **277**, 28; 1979) has reported a polymorphism of human brain polypeptides, and genetic variants of transferrin, recognized by conventional electrophoresis were demonstrated after bidimensional analysis (Anderson *et al.* *Biochem. Biophys. Res. Commun.* **88**, 258; 1979).

In all cases, the variants detected by the bidimensional procedure can be attributed to mutational events leading to a change in charge on the protein molecule. Many of the variants detected by conventional electrophoresis are also attributed to amino acid substitutions leading to a change in net charge and there is good reason to think that the affected residues lie on the surface of the protein. However, this type of charge change variant is more readily detected after electrophoresis of the native protein than of the denatured polypeptides, since in denaturing conditions the frictional resistance of the molecules is greatly increased and the charge change less exposed. Nevertheless, it is interesting to note that Steinberg *et al.* (*Cell* **10**, 381; 1977) have demonstrated that progressive carbamylation of AMP dependent protein kinase leads to discrete shifts in *pI* detectable by the bidimensional procedure, which presumably represent single charge changes. However, one of the problems associated with detecting amino acid modifications by isoelectric focusing is that the efficiency of the resolution, as judged by the relative shift of *pI*, will not be the same over the whole gel, but will depend upon the position in the *pH* gradient and the proportional change in charge.

One way in which this problem could be overcome is by modification of the conditions for bidimensional electrophoresis which is commonly carried out in a broad *pH* gradient and at standard gel concentrations. So far the possibilities for varying these conditions have scarcely been explored and it seems likely that if greater flexibility is introduced then somewhat higher levels of heterozygosity might be revealed.

In contrast, the unidimensional gel electrophoresis method provides many opportunities for manipulating conditions such as *pH*, buffer ions, gel concentrations, solvent polarity, temperature and sample concentration. Effects due to the incorporation of affinity ligands in the gels and interactions with sulphhydryl reagents can also be explored. And it is quite a common experience that analysis of an enzyme in a range of electrophoretic conditions leads to the identification of additional variants and an increased estimate of heterozygosity. On the face of it the unidimensional electrophoretic methods primarily detect

charge change variation, but in practice the procedure almost certainly demonstrates a range of other types of variants which arise as a result of uncharged amino acid replacements. Such variants may be revealed

by differences in affinity of the allozymes for coenzymes, variable interaction with different buffer ions and altered electrophoretic mobility due to conformational distortion. □

Growth hormone: deletions in the protein and introns in the gene

from M. Wallis

MODERN high resolution fractionation methods can reveal that most protein preparations are made up of several different closely-related species. In the case of pituitary growth hormone, such microheterogeneity has a multiplicity of origins, and its detailed analysis has provided a wealth of information about the biochemistry and genetics of the protein. Thus, heterogeneity at the N-terminus of bovine growth hormone is now known to be a consequence of ambiguous processing of the precursor (pregrowth hormone) while heterogeneity at residue 127 reflects an allelic variation in the population of cows from which the hormone is prepared. In the case of human growth hormone various enzymically modified forms of the protein have been described, which may reflect enzymatic processing of the hormone *in vivo*.

Perhaps the most interesting of the many growth hormone variants, however, is a form isolated from human pituitaries and characterized by U.J. Lewis and his colleagues at the Scripps Clinic, La Jolla (*J. Biol. Chem.* **253**, 2679; 1978); this they have called 20K human growth hormone, since it has a molecular weight 1000-2000 less than normal human growth hormone (22K). The 20K variant is smaller because it lacks 15 amino acid residues found in the normal form of the hormone. The precise position of this deletion has now been shown (Lewis, Bonewald & Lewis, *Biochem. Biophys. Res. Commun.* **92**, 511; 1980) to include the entire sequence between positions 32 and 46 (inclusive) of the polypeptide chain; otherwise 20K is identical to the normal form of human growth hormone. The variant possesses growth-promoting activity similar to that of normal human growth hormone, but lacks the insulin-like activity normally associated with the hormone. It exists in all human pituitaries, comprising about 15% of the total growth hormone content.

How is this remarkable variant formed? A clue may be provided by recent work on the structure of the growth hormone gene by H.M. Goodman and his colleagues at the University of California, San Francisco (Fiddes *et al.* *Proc. natn. Acad. Sci. U.S.A.* **76**, 4294; 1979). They have shown that the gene is split and contains at least M. Wallis is in the School of Biological Sciences, University of Sussex.

three intervening sequences (introns). One of these occurs between the codons for residues 31 and 32 in the mRNA — precisely the position of the start of the deletion in 20K. The end of the deleted sequence (between residues 46 and 47) does not correspond to the position of another intervening sequence, but it is noteworthy that the last two bases of the codon for residue 46 (glutamine) are AG (Martial *et al.* *Science* **205**, 602; 1979), a base sequence found almost universally at the 3' end of an intervening sequence in the primary RNA transcript of a gene (Crick *Science* **204**, 264; 1979). It is difficult to believe that this partial correspondence between a deleted sequence in the protein and an intervening sequence in the corresponding gene is a coincidence. It seems likely, therefore, either that 20K represents the product of a duplicated gene in which an intervening sequence has been lengthened to include part of a coding sequence (exon) or that the messenger RNA for 20K is produced from the same primary RNA transcript (and therefore the same gene) as normal human growth hormone, which would therefore be processed in two different ways. In the second case, a region of the primary RNA transcript that is a coding region for normal human growth hormone would be treated as part of an intervening sequence for the 20K variant, and different ways of processing a single primary RNA transcript would lead to production of two different proteins.

Whichever of the two explanations is correct, it is difficult to avoid the conclusion that the occurrence of an intervening sequence in a gene has allowed the formation of a markedly different protein variant. Gilbert (*Nature* **271**, 501; 1978) has proposed that the existence of intervening sequences may allow large changes in protein structure, and this may well be an example of such a change. It is interesting in this connection that the rate of evolution of human growth hormone is much greater than that of the non-primate hormones (Wallis *Biol. Rev.* **52**, 35; 1975). Whether the potential benefit of such large evolutionary jumps is in itself sufficient to explain the occurrence of intervening sequences, or whether other causes led to their origin and the evolutionary jumps are a consequence of their existence, remains to be seen. □

Caenorhabditis Genetics Center

A *Caenorhabditis* Genetics Center (CGC), sponsored by the National Institute on Aging, is being established at the University of Missouri. The CGC will be responsible for acquisition, banking, and distribution of *C. elegans* strains and reference strains of other *Caenorhabditis* species. Related services will include maintenance and annual distribution of the genetic map of *C. elegans*, coordination of genetic nomenclature, and maintenance and distribution of a bibliography of research publications.

The centre is the only one planned for *Caenorhabditis elegans*, an organism that has attracted attention in recent years as a research model for genetics, neurobiology, and developmental biology as well as aging.

The establishment of a *Caenorhabditis* Genetics Center (CGC) will promote the rapid and orderly accumulation and documentation of the genetic wealth of *C. elegans* for the benefit of all types of biomedical research on this organism. The genetics of *C. elegans* already enjoys important advantages over more traditional genetic systems. The ability to freeze stocks permits the reliable preservation of all known mutant types. The ancestry of virtually all strains is known so most stocks can be accurately described. A uniform system of genetic nomenclature has been instituted. Since most genetic data have been accumulated in only a few laboratories, the centralization of these data is feasible.

The specific goals of the CGS are (1) to establish a reliable and accessible genetic stock repository, including represent-

ative mutant alleles of all characterized genes as well as chromosome rearrangements, (2) to coordinate and publicize a uniform genetic nomenclature, (3) to coordinate and publicize the delineation of the genetic map, and (4) to develop a computer-based data storage and retrieval system which will handle bibliographic information and data used to generate the genetic map, as well as descriptive data on mutant strains.

The CGC strain collection will include at least one allele of each identified gene, all available chromosomal rearrangements, and available closely linked double mutants, as well as other strains useful for genetic mapping. Laboratories providing mutants of *C. elegans* will be requested to include such information as name of strain; names of contained mutation(s); mutagen used; whether the strain was backcrossed and, if so, how many times; genes affected by the mutation(s); map location(s) of mutation(s); and data used to determine map locations.

C. elegans strains will be available without cost to all qualified investigators pursuing genetic and/or related studies with *C. elegans*. The Center will not be fully operational until the Fall of 1980, but some services are available now. Inquiries should be addressed to: Dr. Margaret M. Swanson, Curator, or Dr. Donald L. Riddle, Director, *Caenorhabditis* Genetics Center, Division of Biological Sciences, Tucker Hall, University of Missouri, Columbia, Missouri 65211.

Though the types vary considerably, many of the contexts from which they come are of high status, being predominantly rich burials. In addition a number of objects combine iron with a precious metal, such as iron rings with a covering of sheet gold or an iron pin with a gold head. It is clear that iron was being increasingly used in societies with a highly developed competence in metallurgy, frequently but not exclusively in association with objects of high status. Analyses have so far been few, but many of these early iron objects have been considered meteoric on the grounds of their high nickel content. There has been some controversy over objects with a rather lower percentage of nickel, but there can be no doubt that throughout the period, and increasingly towards the later phases, terrestrial ores were also being exploited.

Similar evidence of an early origin for iron-working has also come from other areas. Some of the most exciting archaeological discoveries of recent years have been made at Ban Chang in north-east Thailand. In addition to surprisingly early occurrences of agriculture, irrigation and copper metallurgy, iron was in use by at least 1600 BC. As Gorman and Charoenwongsa (*Expedition* 18, 14; 1976) show, iron was used especially for the blades of bronze-handled weapons found in rich graves. In China, iron was similarly used in a composite bimetallic weapon in a rich grave of the Shang period at K'ao-Cheng, perhaps around 1200 BC. As Chang (*Archaeology of China*, 351; 1977) points out, there too the iron may be of meteoric origin since other comparable weapons, now in western museums, have been proved to be so.

In Europe too, reports of early iron have increased considerably in recent years. Brongers and Woltering (*Prehistorie van Nederland*, 97; 1978) describe finds of iron and slag with radiocarbon dates as early as the 12th century BC, while an iron fragment from a knife found in Slovakia is dated to 1465±35 BC (Butler in *IX Congress UISPP, Résumés*, 431; 1976). Another line of evidence has been followed by Bouzek, who has studied the technology of the decoration of bronzes in the late bronze age (*Zeitschr. f. Archäol.* 12, 9; 1978). He found that iron tools were increasingly used from 1100 BC, a conclusion now supported by the discovery of the appropriate iron tools in contemporary graves. The only detailed regional study is by Laszlo (*Acta Archaeol. Hung.* 29, 53; 1977), who documents recent discoveries in Romania, where iron became progressively more common through the later bronze age from 1200 BC onwards, copying the local bronze forms and even being manufactured in the same workshops as bronze.

All these regions share a number of common features such as the pre-existence of a developed metallurgical technology, the copying of bronze forms in iron, the large proportion of iron objects which are

The early development of iron-working

from T.C. Champion

TRADITIONAL explanations for the adoption of technological innovations in archaeology are being called into question by recent research. Much attention has been paid to the origins of metallurgy, and to the possibility of multiple independent inventions of copper working, but until recently the development of iron working had been relatively ignored. Recent work now suggests that the discovery of iron did not occur in one unique area from which its knowledge was diffused, that early iron was not superior to bronze, and that bronze-using communities did not adopt it immediately. Instead, what has usually been regarded as an obvious technological advance appears, when seen against the background of current economic and social conditions, to be due to much more complex reasons.

The traditional view held that the technology of iron was developed only in the Near East and was guarded as a royal secret. T.C. Champion is in the Department of Archaeology, University of Southampton.

monopoly by the Hittite empire during the second millennium. The collapse of the empire about 1200 BC allowed the knowledge of iron-working to spread to other areas where the new material was progressively adopted as its potential for making superior weapons and tools was appreciated. This view was based as much on the interpretation of Hittite historical documents as on archaeological finds but recent work by Waldbaum (*Stud. Mediterr. Archaeol.* 54, 1; 1978) now shows it to be untenable. Iron was known and used regularly throughout the area of the eastern Mediterranean from the early bronze age onwards and though some finds are known from as early as the beginning of the third millennium they become much more common in the mid-second millennium. Tools, weapons and jewelry are all made in iron. As the forms are in most cases specifically local ones which copy pre-existing bronze examples it would seem that iron was worked locally rather than imported from a single centre.

of high status, and in many cases the use of iron in combination with other metals. A rather different picture is presented in India, where Chakrabarti (*Antiquity* 50, 114; 1976) has argued for an independent growth of iron-working by 800 BC. Here, however, the range of iron goods is predominantly domestic, and a phase of gradual development has yet to be documented. It is possible that the earliest stages of iron-working in the subcontinent have yet to be explored as the date of 1025 ± 110 BC reported by Jain (*Prehistory and Protohistory of India*, 191; 1979) for a layer containing small iron tools would support the suggestion of a more extended development. Even more problematical is the question of the origin of iron in west Africa. The technology described by Tylecote (*W. Afr. J. Archaeol.* 5, 1; 1975) from the Nok culture of Nigeria is already well developed by about the fourth century BC, too early to be derived from any of the known industries of the north-east in Egypt or the Sudan. The solution proposed is that the technology diffused from the north coast across the Sahara but the possibility of independent invention cannot be ruled out. There was no previous tradition of metallurgy but the ready availability of high-grade ores and the pyrotechnic skill demonstrated by the Nok clay figurines fulfil some of the necessary preconditions.

The theory of a unique invention of iron technology is clearly now very difficult, if not impossible, to maintain, and the focus of research has moved from questions of diffusion to the conditions under which the technology developed. Although the west African case still remains problematic, the other areas all shared a competent bronze metallurgy and in most of them the use of early iron is strongly associated with high status objects. The development of iron technology may therefore be seen as taking place in stratified societies where considerable technological resources are invested in producing prestige items. This stage of early development is quite distinct from a later phase when iron replaces bronze for a wide range of everyday items. Snodgrass (*Dark Age of Greece*, 213; 1971) argued, on the basis of limited analyses of early iron objects, that they were in fact not superior to the quality of bronzes but actually inferior and he attributed the wide-scale adoption of iron in Greece to an interruption in the supply of raw materials forcing the use of local resources. Maddin, Muhly and Wheeler (*Scient. Amer.* 237, 122; 1977) have described the discovery of the essential techniques of carburization, quenching and tempering in the Near East, but they too, like Waldbaum, ascribe the demise of bronze to a breakdown in the supply of raw materials, especially tin. Further analyses are needed of both iron and bronze objects, but it is now recognized that the problems of the discovery and adoption of iron can only be understood in relation to contemporary social and economic conditions. □

Ocean trench topography

from D.L. Turcotte

THE anomalous topography and negative gravity anomalies of ocean trenches are readily explained by plate tectonics. The oceanic lithosphere bends and descends into the interior of the Earth in a process referred to as subduction. This sounds relatively straightforward until one realises that the oceanic lithosphere is a 100 km thick plate made up of cool, brittle rock. How can a plate 100 km thick made up of a brittle material be bent through an angle of 45° or more in the subduction process?

Two recent papers examine alternative hypotheses for the bending behaviour of the lithosphere. Chapple and Forsyth (*J. geophys. Res.* 84, 6729; 1979) argue in favour of an elastic-perfectly plastic rheology. In the upper 20 km they assume that the rock is relatively weak due to low lithostatic pressure (the weight of the overlying rock) and postulate a yield stress of 1 kbar. In this region the tensional bending stresses due to the flexure of the lithosphere at an ocean trench give the normal faulting earthquakes observed seaward of many ocean trenches. Between depths of 20 and 50 km a yield stress of 6 kbar is postulated. At stresses less than 6 kbar the rock is assumed to behave elastically and then yields freely when the yield stress is reached. This is the definition of an elastic-perfectly plastic rheology. It is this part of the lithosphere which transmits the high stresses associated with the bending. At depths greater than 50 km temperatures are assumed to be sufficiently high that thermally-activated creep processes (that is, dislocation creep) relieve the elastic stresses. Predicted trench topography agrees well with observations.

The primary objection to the application of the elastic-perfectly plastic rheology is the high stress level required. Many seismologists object to high stresses (greater than a few kilobars) in the lithosphere. Seismic studies indicate that the stress drops in most large earthquakes are of the order of 100 bar or less. Stress fields derived from the surface strain associated with earthquakes give similar values. On the other hand, scientists who study the mechanical behaviour of rock in the laboratory tend to favour high stresses. Mantle rocks tested in the laboratory can easily be stressed to 5 kbar without failure. Extrapolations of laboratory derived friction laws and creep studies also give high stresses in the lithosphere.

An alternative approach to the problem of the bending of the oceanic lithosphere at ocean trenches has recently been given by Melosh and Raetsky (*Geophys. J.* 60, 333; 1980). These authors postulate that the oceanic lithosphere is made up of a near-

surface elastic layer with a thickness of 15–30 km overlying a highly viscous layer 50–80 km thick. The shape of the bending lithosphere is primarily attributed to the viscous flow in the lower lithosphere. The viscous flow is attributed to solid-state creep. Again good agreement with the observations is obtained. The maximum stress required is a few hundred bars.

The principal advantage of the viscous rheology is the lower required stress level. However, the derived viscosities of 2×10^{22} – 10^{23} poise are quite low, being less than an order of magnitude larger than the viscosity associated with solid-state creep in the deeper mantle. This does not seem to be consistent with the temperature dependence of thermally activated creep processes.

It is clear that comparisons with observations at oceanic trenches cannot differentiate between the elastic-perfectly plastic and elastic-viscous hypotheses. The alternative rheologies must be applied to other flexure problems. Examples include the flexure of the lithosphere under the load of oceanic islands, the load of sediments, and the load of mountain belts. It is implicit in the viscous analysis that bending stresses will relax with time. Whether the resultant deformation of the lithosphere when subjected to a fixed load can be observed is also a subject of controversy.

An understanding of the bending behaviour of the lithosphere under load will help in our understanding of how stress is transmitted through it. An essential part of this understanding is the stress level present. At present the stress level at depth in the lithosphere is uncertain by more than an order of magnitude. Only when we understand the state of stress in the lithosphere will we be able to start to understand in some detail why earthquakes occur. □



100 years ago

BAUMGARTNER, the inventor of a navigable balloon, having three cars attached, each with ten or twelve wings, set in motion by a crank, has attempted an ascent at Leipzig. On the rope being cut the balloon rose very slowly, skimming the house-tops, whereupon the two assistants jumped out of the centre car in alarm. The balloon shot up to a great height, then burst and fell. Baumgartner was not seriously hurt, and is resolved on a second experiment.

From *Nature* 21, 8 April, 549; 1880.

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REVIEW ARTICLE

Peptidergic neurones

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More than 20 peptides have been identified in neurones of the brain, spinal cord and periphery. In several cases a peptide occurs together with a 'classical' transmitter in the same neurone. This raises new questions about the process of neurotransmission and may have important implications for our understanding and treatment of mental disorders.

It is generally accepted that nerve impulses are transferred either from one neurone to another or to an effector cell by chemical neurotransmitters released at the neuronal synapses. In 1963, McLennan¹ listed seven chemically identified putative transmitters including acetylcholine (ACh), γ -aminobutyric acid (GABA) and monoamines such as noradrenaline and dopamine. As, crudely speaking, neurotransmitters act in only two ways—to excite or to inhibit the postsynaptic cell—seven candidates seemed more than sufficient. Therefore, the recent discovery that many peptides also seem to act as transmitters in the nervous system^{2,3} has evoked both interest and doubt.

Doubts arise not only about the need for more than 20 peptide neurotransmitters, given that one excitatory and one inhibitory transmitter should be sufficient to operate the nervous system, but also because 'classical' transmitters have always been small molecules (molecular weight ~200), whereas some of the peptides consist of up to 30 or more amino acids (MW ~3,000). Furthermore, many peptides now identified in the brain were previously thought of as peripheral hormones, such as angiotensin II^{4,5} and members of the gastrin/cholecystokinin (CCK) family^{6,7}. Even some of the large pituitary hormones, prolactin, adrenocorticotrophic hormone (ACTH), thyroid stimulating hormone (TSH) and growth hormone, and also the pancreatic hormones insulin and glucagon, may be present in brain (see ref. 8). But the idea of a compound being both a hormone and a neurotransmitter should not be so surprising as we have long accepted such a dual role for noradrenaline⁹.

The realisation that mammalian neurones produced and released peptides followed du Vigneaud's characterisation of oxytocin and vasopressin as octapeptides¹⁰. These two hormones are released into the blood in the posterior pituitary from the end of neurones which originate in the supraoptic and paraventricular nuclei of the hypothalamus. These neurones, previously described by the Scharrers, Bargmann and others¹¹ as neurosecretory, magnocellular neurones, were thus the first peptide neurones identified in the mammalian brain. The next peptide neurones to be discovered were those containing the three hypothalamic hormones, thyrotropin releasing hormone (TRH), luteinising hormone releasing hormone (LHRH), and growth hormone release-inhibiting hormone (somatostatin)^{12,13}. These neurones are also hypothalamic in origin, they also secrete their content into blood vessels and are therefore also neurosecretory. Neuroanatomically these systems have been assumed to correspond to the parvocellular tuberoinfundibular neurones described by Szentagothai *et al.*¹⁴.

The major impetus for the study of neurones containing either the hypothalamic peptide hormones or the host of other chemically identified peptides came when the peptides became available in amounts sufficient for production of antibodies. This made it possible to measure peptides in tissues and body fluids by radioimmunoassay¹⁵ and to localise peptides at

the cellular and ultrastructural level using immunocytochemistry.

It is important to realise that, whereas a transmitter role seems well substantiated for a few peptides, crucial experimental evidence is lacking for many others, and additional functions of neuropeptides, as trophic factors or factors involved in long-term events, should be considered. It is, however, not the aim of this review to discuss the evidence for peptides as transmitters (see refs 16, 17). Instead, we shall concentrate on the morphology and distribution of some peptide systems, as revealed by immunocytochemistry, and what this may tell us of possible functions. Of particular interest has been the discovery of peptides in neurones which also contain a classical transmitter such as noradrenaline, serotonin (5-HT), dopamine or ACh¹⁸. This is a situation similar to the one seen in certain endocrine cells, which belong to the APUD (amine precursor uptake and decarboxylation) system as defined by Pearse¹⁹ and which store both a peptide hormone and a biogenic amine, further underlining the close relationship between the endocrine and nervous systems.

The immunohistochemical technique

Immunohistochemistry²⁰ has evolved as an extremely useful technique for visualising different types of compounds in nervous (and other) tissues²¹⁻²³. In principle any substance can be traced provided that (1) it is available in pure form, (2) it is immunogenic (or can be rendered immunogenic by conjugation to carrier protein) and (3) it can be retained in tissue sections during processing for immunohistochemistry. In addition to this 'universal' applicability, this method has the advantage that it can be combined with other types of histochemical techniques. Furthermore, 'double-staining' techniques^{24,25} allow visualisation of more than one substance in a given section.

With the immunohistochemical approach antisera raised against peptides conjugated to carrier proteins such as bovine serum albumin, are applied to tissue sections and visualised by fluorescent or other types of markers. Various modifications can be used: the classical indirect immunofluorescence technique of Coons and collaborators²⁶; the peroxidase technique²⁷; and the sensitive peroxidase-antiperoxidase (PAP) technique²⁸. Apart from limitations of sensitivity, and the limited ability of the antibodies to penetrate to intracellular storage sites, the major problem is the specificity of the immunological reaction. Thus, an antiserum may not only react with the proper antigen but also cross-react with structurally similar peptides. Hence the cautionary use of terms such as 'substance P immunoreactive' and 'substance P-like immunoreactivity'. Future research may show that, when multiple systems are described with a particular antibody, they are not homogeneous with regard to their peptide content. Thus, for example, of the more than 30 groups of

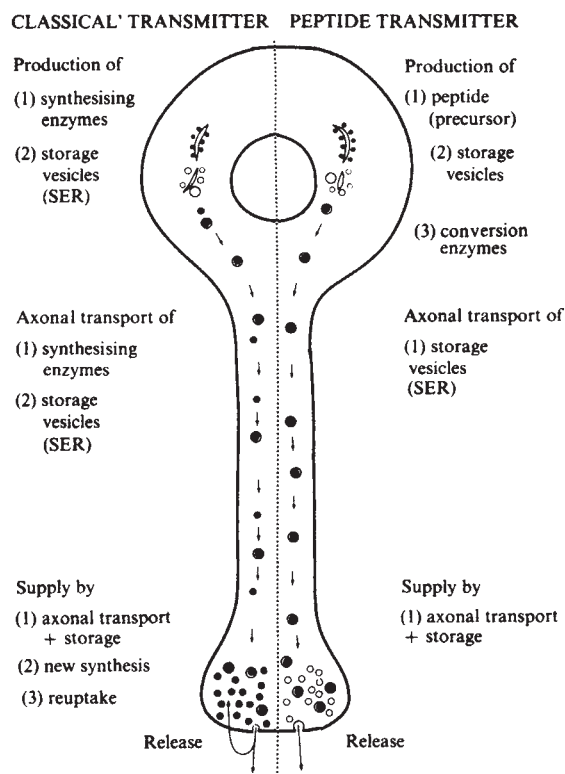


Fig. 1 Schematic drawing of a neurone demonstrating some differences between a neurone using a 'classical' transmitter such as noradrenaline (left) and a peptide transmitter (right). SER, smooth endoplasmic reticulum.

substance P immunoreactive cells found in the rat central nervous system (CNS)²⁹, perhaps only a fraction of them contain the genuine undecapeptide³⁰.

The complexity of this problem can be well illustrated by the opioid peptides³¹⁻⁴⁶. Hughes *et al.*³¹ originally discovered two pentapeptides—Leu- and Met-enkephalin—but by now several other larger opioid peptides have been shown to contain one or other of these pentapeptide sequences³¹⁻³⁶. Using antisera to Leu- and/or Met-enkephalin, many groups have described the central cellular localisation of 'enkephalin' neurones³⁷⁻⁴⁴. Because of the problem with cross-reactivity, it was not possible to decide whether the two pentapeptides were present in separate or in the same neurones, and only recently have Larsson *et al.*⁴⁴ provided evidence that Leu- and Met-enkephalin neurones constitute separate systems. It remains to be established to what extent the 'enkephalin' neurones contain the pentapeptides, as opposed to the large opioid peptides from which, in some cases, they are likely to be derived. It is clear, however, that a separate β -endorphin system exists in the rat brain^{45,46}.

A further problem with the immunocytochemical mapping of peptide neurones comes in visualising peptides in the cell soma and axon. This is probably because they contain peptides either in low concentration or in precursor form. In the latter case an antibody cross-reacting with a precursor is an asset rather than a hindrance. Thus, using one of our somatostatin antisera, cell bodies can easily be identified by means of a strong immunoreaction localised in the Golgi apparatus⁴⁷. This particular antibody is known to cross-react with a probable precursor molecule⁴⁸ and it is tempting to speculate that the Golgi-associated somatostatin-immunoreactive pool represents the precursor. Another approach for visualising peptides in cell somata is the use of colchicine, a drug which inhibits axonal flow^{49,50} and in this way increases peptide levels. Peptides in axons may be detected by ligation of nerves⁵¹ and the resulting accumulation of the peptide.

Some characteristics of peptide neurones

Only limited information is available on the structural, biochemical and physiological properties of peptide neurones. Nevertheless, it seems that they do not have unique characteristics which would allow their identification on pure morphological grounds. Peptide neurones may be small or large, multipolar or pseudo-unipolar, their nerve endings may make morphologically defined synapses or they may act on receptors over 'long' distance and they may have long or short projections. Nor does ultrastructural analysis reveal any fine structural features exclusively characteristic of peptide neurones. One feature that is common to several classes of peptide neurone is the large granular vesicle⁵², a type originally described in peripheral adrenergic neurones⁵³.

Although functional evidence suggests that at least some peptides act as neurotransmitters^{2,3,16,17,54}, the method of replenishment of peptide neurotransmitters to nerve endings seems to be different from that of classical neurotransmitters (this difference is shown schematically in Fig. 1). For example, intraneuronal noradrenaline levels are kept fairly constant by the efficient replacement of released transmitter by (1) enzymatic synthesis in the nerve endings, (2) re-uptake from the extraneuronal (synaptic) space through an active membrane mechanism and (3) supply of amine in storage vesicles (or their precursors) from the cell body via axonal transport⁵⁵. Peptides, on the other hand, are probably produced only on the ribosomes of the cell soma, possibly in the form of a larger precursor molecule⁵⁶ without local synthesis in nerve endings. As no reuptake mechanisms seem to operate for peptides in nerve endings, every single peptide molecule released from a nerve ending must be replaced by axonal transport. This comparatively inefficient and slow mechanism should be reflected in the dynamics of synaptic events at peptide synapses⁵⁷. Perhaps peptides are released intermittently rather than tonically. Such an intermittent release may, however, be 'compensated' for by a long duration of action. The amounts of peptide released may also be much smaller than those of classical transmitters. This would be in line with the rather low concentrations of peptides found in the CNS (~1,000 times less than monoamines and 100,000 times less than amino acids). Furthermore, peptides may activate receptors at much lower concentrations than the classical transmitters, a situation which may compensate for an 'inefficient' replacement of released transmitter (see below).

Distribution of peptide neurones

Early studies both with radioimmunological and immunohistochemical techniques indicated that the three hypothalamic hormones—TRH, LHRH and somatostatin—were present outside the median eminence and even outside the hypothalamus. The finding of these neurosecretory products, previously assumed to be exclusively involved in pituitary control, also in, for example, the spinal cord was followed by reports of the presence of so called gut hormones such as vasoactive intestinal polypeptide (VIP)⁵⁸⁻⁶⁰ and CCK⁶ in many areas of the brain. A hypothesis has since emerged according to which the central and peripheral nervous systems and the endocrine system have a number of peptides, or perhaps families of peptides, in common⁶¹. Although differences can be observed between various species, the occurrence of peptide-containing neurones is widespread.

Central peptide neurones

More or less extensive maps of the many central peptide systems have been published: for immunoreactive substance P^{29,62}, enkephalin³⁷⁻⁴⁴, neurotensin⁶³, CCK⁶⁴⁻⁶⁶ and bradykinin⁶⁷. Although they overlap in many areas, each central peptide system has a unique distribution pattern of cell bodies. For example, although both substance P and enkephalin immunoreactive cell bodies have been described in more than 30 areas of the CNS, they overlap only slightly and, when present in the same nuclei, the cells do not seem to be identical.

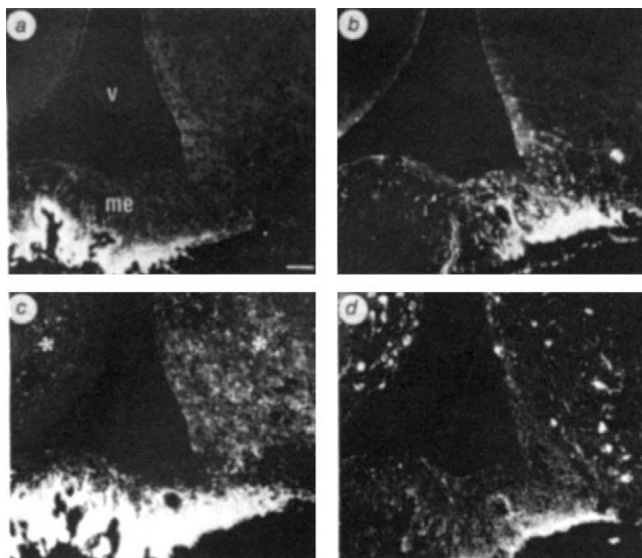


Fig. 2 Immunofluorescence micrographs of four serial sections incubated with antiserum to TRH (a), LHRH (b), somatostatin (c) and tyrosine hydroxylase, a marker for catecholamine neurones (d). All four substances are present in the median eminence (me) close to portal vessels (arrows), but with clearly different distribution patterns indicating their occurrence in separate neurone systems. Note dense plexus of somatostatin in nerve fibres also outside the median eminence in the arcuate nucleus (asterisks) suggesting a transmitter role. Of these four systems only the dopamine neurones have their cell bodies in the basal hypothalamus (d). V, ventricular. Scale bar, 50 μ m (from ref. 181).

Coexistence of two peptides has, however, been described. Immunoreactive ACTH and β -endorphin are present in the same neurones in the hypothalamus⁶⁸⁻⁷⁰, and recently we have observed TRH-like immunoreactivity in substance P (and 5-HT) immunoreactive neurones of the medulla oblongata^{18,71}.

It is clear from the data collected so far, that some areas of the brain are rich and others are poor in peptide-containing neurones. The hypothalamus, the amygdaloid complex (particularly its central nucleus), certain nuclei of the medulla oblongata and the dorsal horn of the spinal cord are examples of areas of the rat brain where most peptides can be found. In the cerebellum, on the other hand, only scattered peptide fibres are observed. The cerebral cortex seems to lack many peptides but is rich in somatostatin-, VIP- and a gastrin/CCK-like peptides, all present in interneurons⁷². Of particular interest are the median eminence of the hypothalamus and the dorsal horn of the spinal cord.

The first immunohistochemical identification of a small peptide in neurones was of LHRH-immunoreactive fibres observed in the external layer of the median eminence of the hypothalamus⁷³⁻⁷⁵. TRH and somatostatin fibres were also later found in this brain region (Fig. 2). Lesion studies suggest that these three peptides do not originate in the basal hypothalamus, as assumed originally, but deeper within the hypothalamus⁷⁶. Instead, the arcuate neurones with short projections to the median eminence seem to contain small classical transmitters such as dopamine⁷⁷, GABA⁷⁸ or ACh⁷⁹, which may exert part of their action at the median eminence level rather than being released into portal vessels. It may be, therefore, that a set of short tuberoinfundibular dopamine, GABA and ACh neurones act as a type of 'gating system', controlling the release of hypothalamic hormones into the portal vessels via an axo-axonic influence⁷⁶.

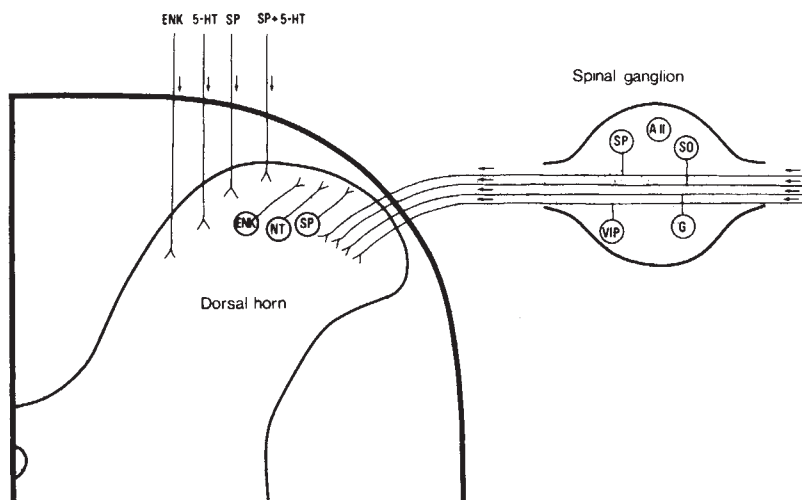
The dorsal horn of the spinal cord receives a complex input of peptide-containing neurones as seen in Fig. 3. Immunoreactive substance P, somatostatin, VIP and gastrin/CCK are present in primary sensory neurones^{65,80,81}. Substance P (and perhaps somatostatin) is also observed in propriospinal neurones or spinal interneurons^{29,82}. Whether the supraspinal substance P/5-HT neurones, which descend to the ventral horn⁸³, also project to the dorsal horn is not yet firmly established. A descending enkephalin-immunoreactive system⁸⁴ may also project to the dorsal horn. Neurotensin⁶⁴ and enkephalin^{38,85} are present in interneurons (or possibly propriospinal neurones). These findings suggest that peptides play an important part in the processing of sensory information at the spinal level.

Peripheral and sensory peptide neurones

The wide distribution of peptides in peripheral neurones has now been firmly established. Initial observations concerned substance P in primary sensory neurones⁸⁶⁻⁸⁸, but this and other peptides are also present in virtually all parts of the autonomic nervous system, particularly the gut and certain autonomic ganglia. There are well documented examples of primary sensory neurones containing immunoreactive substance P, somatostatin⁸⁹, VIP⁸¹ and gastrin/CCK^{65,81}. It is not yet absolutely clear whether these peptides can ever occur in the same neurones, but substance P and somatostatin at least do not⁸⁹. Substance P-like immunoreactivity has also been observed in the vagus nerve^{81,90-94} and in taste neurones of the cat⁹⁵. Thus, substance P may be involved in sensory processes of various types, including pain⁹⁶.

If substance P is a transmitter substance in primary sensory neurones one might expect its main action to be exerted at the central branches in the spinal cord or brain stem. A role for

Fig. 3 Schematic illustration of a spinal ganglion and the dorsal horn of the spinal cord and some of its peptide systems. Note that substance P (SP) probably is present in three, possibly four different systems: (1) primary sensory neurones; (2) interneurons or propriospinal neurones; (3) descending systems, some of which may contain both SP and 5-HT. AII, angiotensin II; ENK, enkephalin; G, gastrin/CCK; NT, neurotensin; SO, somatostatin; VIP, vasoactive intestinal polypeptide. In the dorsal horn there are other peptide and non-peptide systems, not mentioned here (from ref. 80).



substance P in the periphery has, however, long been suspected, for example in the axon reflex inducing antidromic vasodilation⁹⁶⁻⁹⁸. Recently, Olgarth *et al.*⁹⁹ have demonstrated that substance P can be released from peripheral, presumably sensory, branches in the tooth pulp. Interestingly, more than 90% of the substance P immunoreactive material produced in the nodose ganglion (of the vagus system) seems to be transported into the peripheral branch⁹⁴. All these findings suggest that substance P may have an important function at its sensory terminal branches by, in fact, acting at 'postsynaptic' cells or perhaps by modulating the threshold of 'its own' nerve ending. The occurrence of substance P in presumptive taste neurones also indicates other possibilities. It is well known that the structural integrity of the taste buds is dependent on their sensory innervation¹⁰⁰. Transplantation experiments have shown that different types of sensory ganglia, even lumbar sensory ganglia, can induce taste bud formation^{101,102}. In all these ganglia substance P is present. Could it be that this peptide is of trophic importance for the structural integrity of the taste buds?

Autonomic ganglia: Autonomic ganglia, particularly prevertebral sympathetic ganglia in the guinea-pig, contain networks of peptide immunoreactive fibres (Fig. 4). Thus, the inferior mesenteric ganglion has dense plexuses of enkephalin¹⁰³ and VIP¹⁰⁴ immunoreactive fibres and in addition substance P¹⁰⁵, gastrin/CCK and bombesin (ref. 65 and our studies, in preparation) positive fibres are present. A few somatostatin-immunoreactive fibres are also seen¹⁰⁶. Ligation and transection experiments have revealed that some, at least, of these peptides are present in separate systems (ref. 80 and Lundberg *et al.*, in preparation). Thus the enkephalin-immunoreactive fibres

Table 1 Examples of neurones and endocrine cells containing both a classical transmitter and a peptide

Classical transmitter	Peptide	Area (species)	Refs
Serotonin	Substance P	Medulla oblongata (rat)	83, 134-137
Serotonin	TRH	Medulla oblongata (rat)	18, 71
Serotonin	Substance P + TRH	Medulla oblongata (rat)	18, 71
Noradrenaline	Somatostatin	Sympathetic ganglia (guinea pig)	106
Noradrenaline	Enkephalin	SIF-cells (cat)	138
Noradrenaline	Enkephalin	Sup. cerv. ganglion (rat)	103
Noradrenaline	Enkephalin	Adrenal medulla (many species)	138, 139
Noradrenaline	Enkephalin	SIF-cells (guinea pig, cat)	103, 138
Noradrenaline	Neurotensin	Adrenal medulla (cat)	138
Noradrenaline	Somatostatin	Adrenal medulla (man)	140
Adrenaline	Enkephalin	Adrenal medulla (many species)	138-140
Dopamine	Enkephalin	Carotid body (cat, dog, monkey)	141
Dopamine	CCK	Ventral tegmental area (A9, A10) (rat, human)	18, 142
Acetylcholine	VIP	Autonomic ganglia (exocrine glands) (cat)	143, 144

sympathetic prevertebral neurones. Of particular interest are the presumably sensory fibres containing substance P, which may represent collaterals from axons on their way to the gut. A similar arrangement has been proposed for the nervous supply of the nasal mucosa¹⁰⁹. If substance P is released from such collaterals, this could explain the need for a considerable transport of substance P into the peripheral branches of the sensory neurones (see above). In this context it is of interest that Krier¹¹⁰ has demonstrated that substance P increases the excitability of nerve cells in certain sympathetic ganglia.

Gastro-intestinal tract: Peptide neurones containing immunoreactive substance P¹¹¹⁻¹¹⁴, VIP¹¹⁴⁻¹¹⁶, somatostatin^{114,117,118}, enkephalin^{37,114,119,120}, neurotensin¹¹⁴, gastrin/CCK^{65,114} and bombesin¹²¹ have been identified in the gastro-intestinal tract. Analysis of neurones stained consecutively with different antibodies has revealed that substance P, VIP and enkephalin are present in separate systems¹¹⁴. In the proximal colon the somatostatin- and gastrin/CCK-like peptides coexist in some neurones, although separate somatostatin and gastrin/CCK neurones seem to exist¹¹⁴. Most of these peptide systems are intrinsic to the gut¹¹⁴, but morphological and functional analysis is complicated by the fact that the same peptides are also present in extrinsic neurones of, for example, the vagus nerve.

Tracing peptide pathways

Although the localisation of peptide-containing cell bodies and nerve terminals is now well documented, comparatively little is known about the connecting axons because their peptide content is usually too low to detect. Similar problems with catecholamine axons were solved by transection experiments in which the pile up of transmitter on the cell body side of the lesion allowed mapping of the axon pathways¹²². Even so, a very large number of consecutive sections must be processed in order to follow long pathways, for example, from the lower brain stem to the forebrain. Anterograde and retrograde tracing techniques¹²³ helped overcome this limitation. In particular, retrograde peroxidase tracing^{124,125} combined with immunochemical 'staining' with antiserum to tyrosine hydroxylase¹²⁶ has enabled the projection of dopaminergic neurones to be mapped. More recently we have combined retrograde fluorescent dye tracing¹²⁷ with peptide immunohistochemistry¹²⁸ to map, for example, an

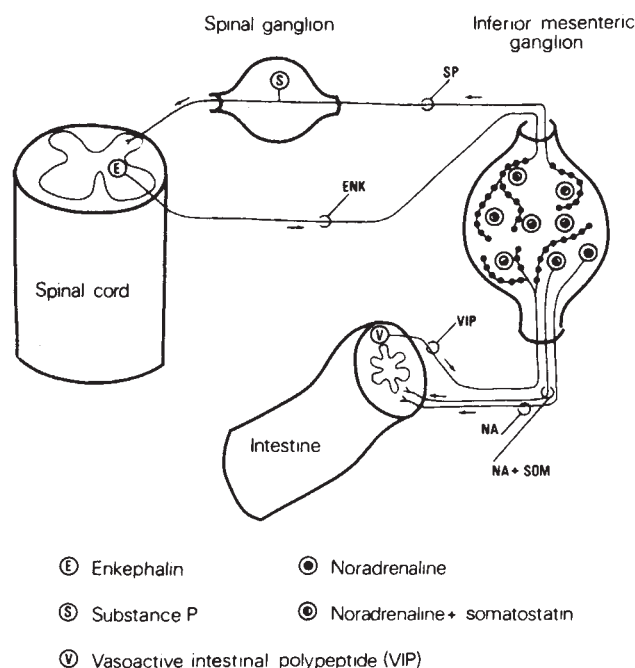


Fig. 4 Schematic illustration of some peptide systems connecting the inferior mesenteric ganglion with other parts of the nervous systems (guinea pig). There may in addition be a gastrin/CCK projection from the gut to the ganglion (from ref. 80).

represent preganglionic neurones, the substance P-immunoreactive ones are of a sensory nature, the VIP and gastrin/CCK fibres probably originate in the gastro-intestinal tract, whereas the somatostatin fibres have their origin within the ganglion itself. Some peptide projections could very well correspond to neurones first described by Kuntz¹⁰⁷ and recently investigated by Kreulen and Szurszewski¹⁰⁸ and assumed to be involved in reflexes between enteric mechanoreceptors and

enkephalin-immunoreactive pathway from the lower medulla oblongata to the spinal cord⁸⁴

Coexistence of peptides and 'classical' transmitters

The coexistence of peptides and amines is well known in endocrine cells, particularly in the gastro-intestinal tract^{19,129-131}. According to Pearse, these cells belong to the APUD system, the cells of which are "neuroendocrine-programmed cells originating from the ectoblast"^{19,132,133}. As neurones have the same embryonic origin it was postulated that they also may contain both a peptide and an amine. The fact that several examples have been observed in a relatively short time (Table 1) may indicate the generality of this situation^{18,71,83,103,106,134-144}. Further examples may be found when new techniques or new markers for other types of neurones have been developed. If so the apparent wealth of 'new' peripheral (and central) peptide neurones may, in fact, only reflect 'old' systems, which in addition to their classical transmitter contain a peptide. Perhaps it will turn out that there are no neurones that contain 'only' peptides.

The possible occurrence of two transmitters in the same nerve terminals raises the question of their subcellular storage sites (Fig. 5). In most types of nerve endings, small (diameter about 500 Å) and large (diameter about 1,000 Å) granular vesicles can be distinguished. Ultrastructural immunocytochemical studies indicate that peptides are usually present in the large vesicles⁵². No ultrastructural immunocytochemical studies have been performed on nerve endings identified and characterised as containing both a peptide and an amine (or ACh). It may, however, be speculated that, as monoamines seem to be present both in small and large vesicles^{145,146}, peptides and amines may coexist in the large vesicles, whereas the small ones may contain only the amine¹⁴⁷. A differential subcellular storage compartment is, however, suggested by the fact that even very high doses of reserpine, which almost completely deplete the monoamine stores, do not seem to affect substance P levels^{83,148}.

It has generally been assumed that one neurone produces and releases only one transmitter, a concept often referred to as Dale's principle^{149,150}. It seems, therefore, important to understand the possible functional significance of two putative transmitters in one neurone. Note that coexistence does not follow a simple pattern. For example, substance P and gastrin/CCK is present only in a small proportion of the 5-HT and dopamine neurones, respectively, originally described by Dahlström and Fuxe¹⁵¹. Similarly, we have observed many more substance P-immunoreactive cell groups (about 30)²⁹ than those containing both substance P and 5-HT. In no case do all of the neurones that contain a particular classical neurotransmitter also contain the same peptide and vice versa. Several explanations seem possible, but an attractive hypothesis is that each type of neurone, defined by the classical neurotransmitter that it

contains, is subdivided into groups characterised by the peptide they contain. We and many others have, in the analysis of the monoamine neurones, previously been struck by their apparent widespread distribution and homogeneity. But perhaps there is really a heterogeneity in which the specific peptides or each subgroup confers the ability to convey differentiated messages.

The best neurones so far available for a functional analysis seem to be those that contain VIP-like immunoreactivity, whilst being rich in acetylcholinesterase (AChE)^{143,144}. Such neurones were first observed in lumbosacral, sympathetic ganglia¹⁴³, which contain one of the best defined cholinergic (AChE-rich and choline acetylase-containing) neurone populations and are assumed to be involved in the regulation of sweat secretion¹⁵²⁻¹⁵⁴. More recent evidence, however, suggests that VIP in cholinergic neurones may be a general characteristic of secretomotor neurones¹⁴⁴. In Fig. 6 a hypothesis for the physiological significance of such neurones is presented. It is generally accepted that ACh produces secretion by an action on the secretory cells (directly and/or indirectly via myoepithelial cells) and that this effect is atropine-sensitive^{152,155}. The concomitant vasodilation, which occurs on stimulation of the postganglionic nerves, on the other hand, is atropine-resistant¹⁵⁵⁻¹⁵⁷. On the basis of studies on colon Fahrenkrug *et al.*¹⁵⁸ have suggested that VIP may be the mediator of atropine-resistant vasodilation. It is now postulated that these two effects—the secretion and vasodilation in exocrine glands—are induced by ACh and VIP, respectively, released from the same nerve terminals to act synergistically^{143,144}. In this particular situation the two putative transmitters would thus act on receptors located on two separate cell types. Clearly, in other systems different mechanisms may operate. There is, for example, evidence that substance P may block cholinergic receptors¹⁵⁹⁻¹⁶¹. In addition, preliminary studies indicate an interaction between 5-HT and substance P at the same receptor in the spinal cord¹⁶², which would agree with the existence of nerve terminals in the spinal cord containing both these compounds⁸³.

Just as the APUD concept has been of importance for our understanding of pathological processes, for example endocrine tumours (so called apudomas) in the gut¹⁶³, so may the coexistence of transmitters be to our understanding of neurological diseases and their treatment. Of particular interest in this respect is the occurrence of a CCK-like peptide in a subpopulation of dopamine neurones in rat and man^{18,142}. In the rat these cells are located mainly in the most medial parts of the zona compacta of the substantia nigra (A9 group)¹⁵¹ and in the ventral tegmental area (A10 group)¹⁵¹ and are known to project mainly to limbic areas in the forebrain¹⁶⁴. In agreement, CCK-immunoreactive (and dopamine-containing) nerve endings are found, for example, in the nucleus accumbens and the tuberculum olfactorium¹⁴².

The occurrence of a peptide in a population of dopamine neurones is interesting in view of our extensive knowledge of the functional role of these dopamine systems. In particular, the mesolimbic dopamine systems have been associated with higher mental functions and, according to the so called dopamine hypothesis, disturbances in this system may represent one component in the pathogenesis of schizophrenia¹⁶⁵. If a CCK-like peptide is released together with dopamine, the peptide could also be involved in the aetiology and symptomatology of schizophrenia. It has recently been demonstrated that CCK fragments can inhibit dopamine release in the nucleus accumbens-tuberculum olfactorium region¹⁶⁶. Thus, the peptide released together with the 'main' transmitter dopamine may be part of an inhibitory feedback system modulating dopamine release via action on autoreceptors. We have speculated¹⁶⁶ that an imbalance between peptide and amine, in fact, could be an aetiological factor for schizophrenia, whereby a loss or decrease in peptide would lead to an 'overactive' dopamine system¹⁶⁵.

Finally, the coexistence of putative peptides and biogenic amine transmitters raises questions about substitution therapy. The belief that some mental and neurological disorders are due to degeneration of transmitter-characterised systems has

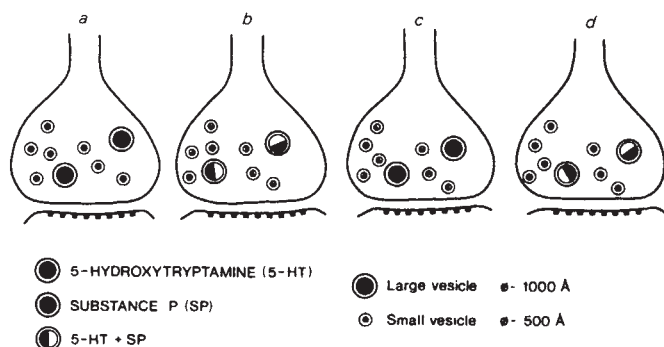


Fig. 5 Schematic illustration of four alternatives for possible storage sites of an amine (5-HT) and a peptide (substance P) coexisting in a nerve ending (From ref. 80).

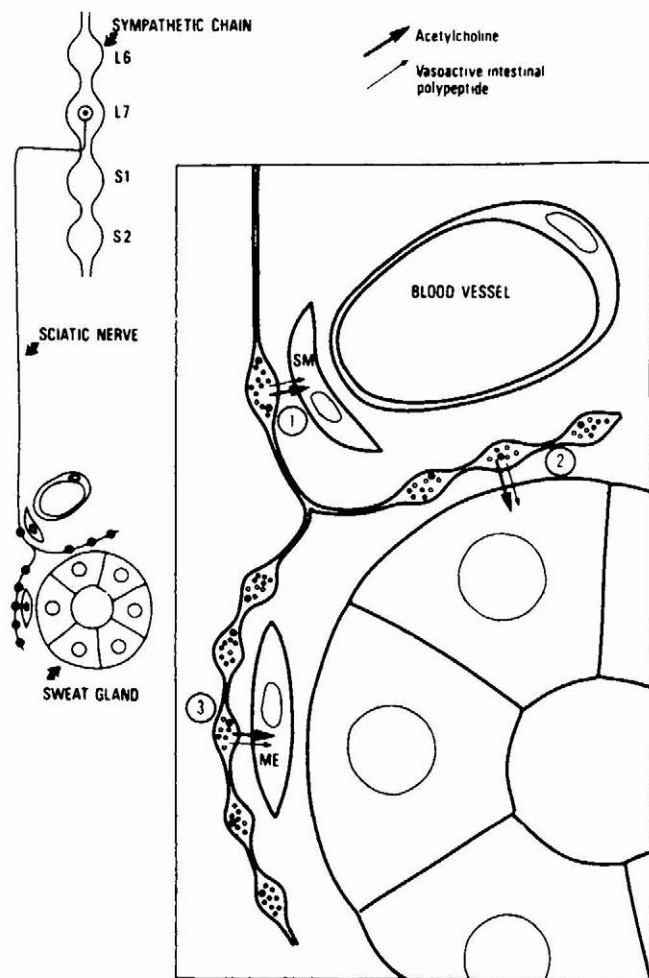


Fig. 6 Schematic illustration of our working hypothesis on the sympathetic innervation of sweat glands. Acetylcholine (thick arrow) and VIP (thin arrow) are released from the same nerve endings. Whereas VIP mainly causes vasodilation by relaxing smooth muscle cells (SM) around blood vessels (1), ACh mainly causes secretion by direct stimulation of secretory cells (2) or indirectly through myoepithelial cells (ME) (3) (from ref. 143).

formed the basis of therapy by administration of the lost transmitter (or its precursor). The best example is Parkinson's disease which is now, due to the pioneering work of Carlsson, Cotzias and Hornykiewicz and others¹⁶⁷, known to be due to degeneration of dopamine systems in the basal ganglia and which can be treated by peripheral administration of L-dopa, the precursor of dopamine. It is obvious that if a neurone releases two transmitters, substitution should, at least in certain conditions, consequently occur with both substances. It may therefore be that future substitution therapy will have to consider administration of two or more components. This argument is, however, not valid if the peptide acts on a presynaptic receptor, as discussed above for the limbic dopamine/CCK neurones. For this particular system other aspects must be considered in relation to drug therapy. Schizophrenia is routinely treated with neuroleptics assumed to exert their beneficial effects via blockade of dopamine receptors¹⁶⁸. It may be that 'dopamine overactivity' can be reduced also by administration of CCK fragments or drugs acting on the hypothetical CCK receptors on dopamine nerve endings¹⁶⁶.

Conclusion

Although our understanding of the functional role of peptides is still incomplete, interesting discoveries have been made. For example, the posterior pituitary hormones seem to be involved in memory processes¹⁶⁹, central endorphins are important in

pain mechanisms¹⁷⁰, substance P in primary sensory neurones may be involved in processing of pain and in axon reflexes causing vasodilation in skin⁹⁶, CCK octapeptide causes satiety¹⁷¹, angiotensin II injected intracerebrally stimulates drinking and increases blood pressure^{172,173} and LHRH increases sexual behaviour¹⁷⁴. The effects mentioned above represent a narrow selection of the work going on in the field of neuropeptide physiology but illustrate the wide variety of functions in which peptides may be involved. In parallel work our knowledge of the electrophysiological characteristics of peptide neurones is rapidly advancing¹⁷⁵⁻¹⁷⁷.

Finally, peptides have been identified in the brain, spinal cord and periphery. Several of these peptides were already known as peripheral hormones. Thus, one and the same peptide, can be present in intestinal endocrine cells, gut neurones, sensory neurones of various types and in a large number of central systems. This may be a way of 'economising' on genetic material. At present the identification of the intraneuronal peptides rests mainly on immunological techniques. But new techniques mean that it will soon be possible to chemically characterise very small samples of peptides. The 'peptide maps' so far published may then have to be revised. Sometimes, or maybe even always, these peptides are found in neurones which also contain one of the classical transmitters, such as a catecholamine, 5-HT or ACh.

There is experimental evidence that at least some of these peptides, for example substance P, act as neurotransmitters. However, comparatively little is known about their exact physiological role. This is partly due to a lack of drugs which can influence peptide induced events at synapses. The wealth of such drugs discovered and developed for studies of monoaminergic mechanisms has been an important reason for the rapid advancement of our understanding of the roles of dopamine, noradrenaline and 5-HT neurones in the brain in normal and pathological conditions^{178,179}. Whilst awaiting the development of equivalent drugs for peptides, some results can be expected from the use of antisera which block peptide-induced effects^{144,180}.

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ARTICLES

Diffusion of light hydrocarbons through near-surface rocks

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The sharply decreasing concentrations for individual light hydrocarbons over the near-surface intervals from several organic matter-bearing shale outcrops indicate that diffusion towards the lithosphere/atmosphere interface is a continuous process. Diffusion coefficients calculated are between 10^{-6} and 10^{-8} cm² s⁻¹ for propane to heptane-range hydrocarbons.

ORGANIC matter in sedimentary rocks which occurs primarily in a solid finely disseminated condition¹ is often very inhomogeneously distributed by interbedded rich and lean strata. Molecular diffusion of light hydrocarbons, generated by burial and temperature increase, through water saturated rock pore spaces should, therefore, be widespread in those sedimentary sequences. Diffusion of propane to heptane-range hydrocarbons through the caprock of a reservoir oil accumulation has been observed². However, except for experimental measurements³ no diffusion rate data are available for light hydrocarbons through rocks in natural conditions; this is partially due to inadequate subsurface sampling. Diffusion of light hydrocarbons is also most likely to occur at the exposed surface of an organic matter-rich shale which, before uplift, had been buried deep enough for hydrocarbon generation. Diffusion processes should be active within the near-surface interval along concentration gradients towards the interface between lithosphere and atmosphere.

Evidence is presented here that light hydrocarbons continuously migrate by molecular diffusion through near-surface intervals of source bed-type shales and ultimately escape into the atmosphere. A mathematical model is then used to calculate diffusion coefficients for individual normal and branched alkanes in the molecular range C₂–C₇. This, to our knowledge, is the first case where absolute rates of diffusion of these light hydrocarbons through rocks were determined in natural conditions. The geochemical significance of these diffusion coefficients is shown by the amount of petrogenic hydrocarbons which return into the global carbon cycle by continuous slow degassing of the lithosphere.

Samples and methods

For this study 38 samples were taken at close intervals from cores of three shallow boreholes (6.70, 8.10 and 10.0 m deep) drilled vertically into outcrops of source bed-type shales. Each core hole was spudded in fresh outcrop, that is any soil, unconsolidated overburden and weathering debris were removed. Core holes B and E were drilled into outcrops of dark grey, silty shales of Upper Cretaceous Campanian/Maastrichtian age at Itivdle and Niaqorssuaq on Nûgssuaq Peninsula, West Greenland. Core hole H penetrated dark grey, calcareous shales of Lower Jurassic Pliensbachian age at an abandoned quarry near Jöllenbeck, North-west Germany. Well consolidated shales of low porosity and permeability which showed no visible traces of weathering alterations of the minerals were found throughout the total length of each core except for a thin, nearly horizontal fracture at 7.3 m depth location H. For a 5-cm thick interval on either side of this fracture the walls of which were stained by iron oxides, the shale showed a pronounced colour change to light grey. A sample was analysed both from this zone and the unweathered dark shale 10 cm below. An outcrop sample from 0.3 m depth was also obtained at each drill site by normal digging. All samples were sealed immediately in

gas-tight tin cans on core retrieval and were stored frozen in the laboratory.

Low molecular weight hydrocarbons were analysed by hydrogen stripping combined with capillary gas chromatography⁴. A glass tube containing a freshly crushed rock sample is placed between the capillary column and the carrier gas supply. Then the sample is stripped by a measured volume of the carrier gas (hydrogen) at ambient temperature. After intermediate trapping the volatile compounds are transported by the carrier gas into the chromatograph by splitless introduction. Detailed compositional information including identification of ~32 individual hydrocarbons in the molecular range C₂–C₈ is obtained from a 1-g sample. This new method is very sensitive, for example, the lowest detectable quantity for *n*-butane is 10⁻⁹% w/w sediment. The kerogen quality was analysed by a pyrolysis technique⁵.

All three shale units contain significant concentrations of organic carbon (2.7, 6.1 and 1.0% mean for core holes B, E and H respectively) and exhibit a narrow range of variation around the mean. Similarly, the kerogen in samples of all three sites is uniformly deficient in hydrogen indicating a type III quality⁶. Mean vitrinite reflectance values of 1.30, 0.98 and 1.74% for sites B, E and H, respectively, indicate that these shale units had adequate subsurface temperatures for hydrocarbon generation. The main difference between these core holes concerns the physical state of the pore water. For sites B and E, West Greenland, the presence of permafrost and thus ice filling the rock pore space can be inferred from their high latitude geographic location.

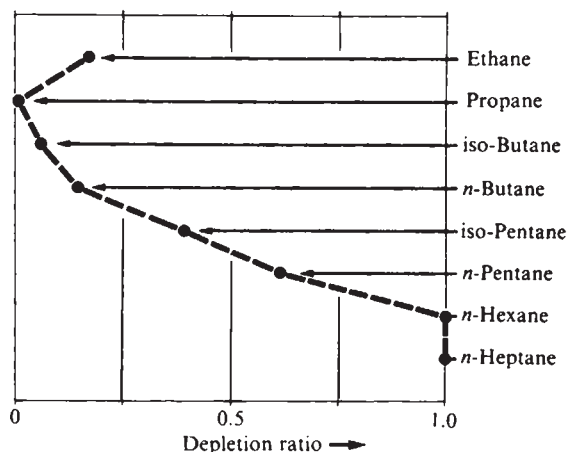


Fig. 1 Depletion ratio for selected light alkanes across near-surface interval of Campanian/Maastrichtian shale outcrop at core hole E, Niaqorssuaq, Nûgssuaq Peninsula, West Greenland. Depletion ratio is defined as the ratio of the sample concentration at 0.3 m to the mean concentration of all samples below 3 m depth.

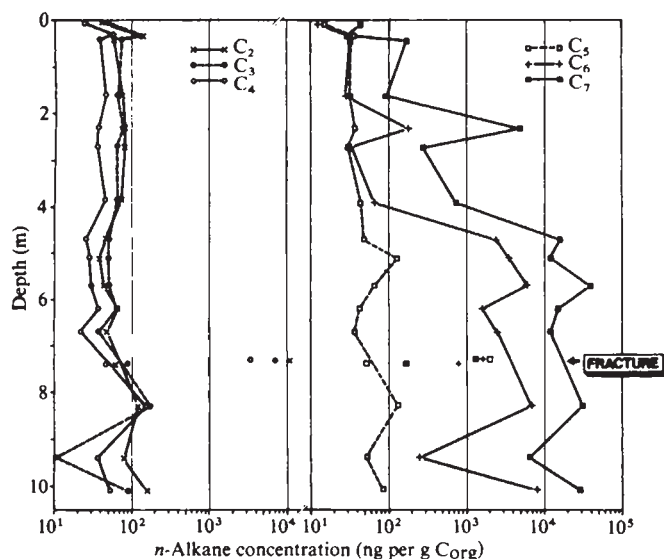


Fig. 2 Depth-plots of *n*-alkane concentrations (ng per g C_{org}) for core hole H, Pliensbachian shale, Jöllenbeck, North-west Germany. Note anomalous values for two samples close to major fracture at 7.3 m depth.

Distribution of light hydrocarbons in near-surface strata

Characteristic depth trends of individual light hydrocarbon (C_2 – C_8) concentrations in the Campanian/Maastrichtian age shales from West Greenland (core holes B and E) suggested that upward diffusion of ethane to pentane-range hydrocarbons was an active process within the near-surface 3-m interval. In particular, regular trends of sharply decreasing concentrations approaching the outcrop surface were recognised indicating a near-surface depletion zone. Details have been published elsewhere⁷. Figure 1 summarises the principal conclusions using core hole E as an example. Below a depth of 3 m a nearly constant concentration was observed for most hydrocarbons. The mean of all samples analysed from below that depth was considered to be the original generated concentration for this shale and is, therefore, assigned a value of unity in Fig. 1. To illustrate the degree of depletion of the sample closest to the outcrop surface, the concentration ratio 'outcrop sample/mean subsurface samples' is termed 'depletion ratio' and shown on the horizontal axis of Fig. 1. The outcrop sample of this shale is most drastically depleted in propane amounting to a 99.8% loss of the original concentration. The diffusive loss decreases drastically with increasing molecular size and also seems (except for ethane) to be controlled by molecular geometry. For example, the *n*-butane diffusive loss is 85.6% compared with 38.9% for *n*-pentane. Diffusion rates of each branched alkane are generally greater than that of the straight-chain isomer. Concentrations of *n*-hexane and *n*-heptane remain nearly constant with depth indicating no, or insignificant, diffusive loss.

Permafrost in West Greenland means that the physical conditions for light hydrocarbon diffusion through near-surface strata are different from those in North-west Germany, where the pore filling is liquid water. Figure 2 shows for core hole H, North-west Germany concentrations of *n*-alkanes ethane to heptane as a function of depth. With respect to the type and magnitude of the concentration changes, the molecular range can clearly be subdivided: for ethane to *n*-butane, comparatively small-scale concentration changes occur (most samples between 20 and 100 ng per g C_{org}), and seem to be independent of the distance from the outcrop surface. However, concentrations of *n*-hexane and *n*-heptane decrease sharply towards the exposure

surface and within the near-surface 5-m interval. The concentration depth pattern of *n*-pentane is between these two hydrocarbon groups. Only samples from depths of 7.3 and 7.4 m are exceptional and will be discussed separately.

For concentrations of *n*-hexane and *n*-heptane two depth intervals can be differentiated in core hole H: below a depth of ~5 m concentrations remain high and vary in a random fashion with depth, whereas in the shallow portion above they decrease continuously and by several orders of magnitude towards the outcrop surface. For example, most samples below 5 m depth have *n*-heptane concentrations between 15,000 and 30,000 ng per g C_{org} , whereas the sample from 0.3 m depth has only 20 ng per g C_{org} . As the molecular size increases this concentration decrease towards the outcrop surface covers a progressively greater range. For *n*-pentane it is <1, and it reaches ~2.5 orders of magnitude for *n*-heptane. Compared with these drastic concentration changes, those for ethane, propane and *n*-butane are minor.

This sharp decrease in concentration for *n*-pentane to *n*-heptane is interpreted to reflect an ~5-m thick near-surface interval where this shale has lost part of its original light hydrocarbon content into the atmosphere. An alternative explanation of an *in situ* origin of these depth concentration trends assuming different generation rates for these light hydrocarbons in the shallower than in the deeper portion of this core, can be dismissed. Concentration changes of this magnitude and over such short intervals could only result from a major change in hydrogen content of the kerogen^{2,7}. However, as discussed above, kerogen type is uniformly deficient in hydrogen

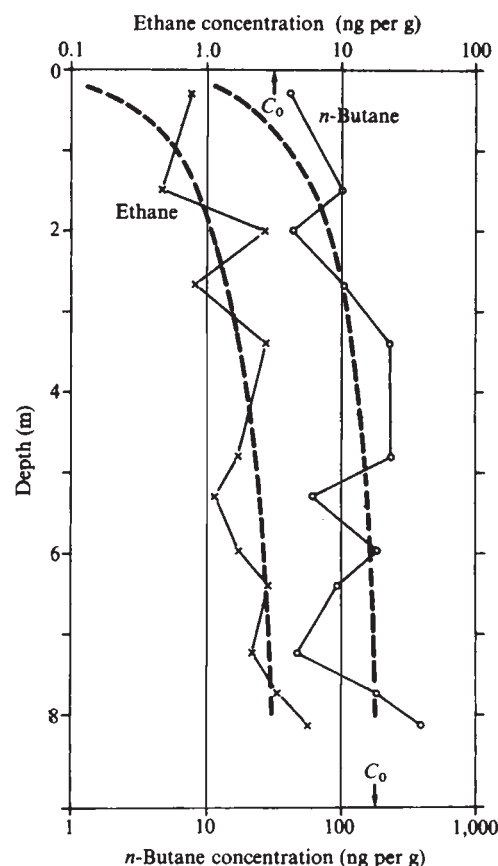


Fig. 3 Computed (broken line) and measured concentration-depth trends for selected *n*-alkanes from core hole E, Campanian/Maastrichtian shale, Niaqorssuaq, Nûgssuaq Peninsula, West Greenland. Initial concentrations C_0 used for calculation of diffusion coefficients for ethane and *n*-butane (3.1 ng per g and 165.0 ng per g respectively) are indicated by arrows.

Table 1 Absolute values of the computed diffusion coefficients ($\text{cm}^2 \text{s}^{-1}$) for selected low molecular weight alkanes in Upper Cretaceous shales from West Greenland

Core hole	Methane	Ethane	Propane	iso-Butane	n-Butane	iso-Pentane	n-Pentane	n-Hexane	n-Heptane
B	—	*	*	3.9×10^{-7}	3.5×10^{-7}	2.7×10^{-7}	1.4×10^{-7}	9.5×10^{-8}	4.2×10^{-8}
E	—	6.4×10^{-7}	1.6×10^{-6}	3.6×10^{-7}	3.2×10^{-7}	2.0×10^{-7}	1.1×10^{-7}	†	†
Recent sediments ¹⁰	3.0×10^{-6}	—	—	—	—	—	—	—	—

* Initial concentration C_0 could not be defined based on available concentration depth trend.

† Insignificant slope of concentration–depth trend towards outcrop surface.

throughout core hole H. Furthermore, a change in kerogen-type would also have to be reflected in the ethane to butane concentrations.

The rate of light hydrocarbon depletion in the shallow 5-m interval increases with molecular size producing pronounced compositional fractionation. This suggests that diffusion is the mechanism by which *n*-pentane, *n*-hexane and *n*-heptane have escaped from the near-surface 5-m interval of this shale. However, the compound separation effects in the Pliensbachian shale of core hole H are with respect to their relationship with molecular size, opposite to what would be expected from simple diffusion through porous media. The degree of depletion in the near-surface interval should be most severe for the gases and decrease with molecular size. The lack of a measurable concentration gradient for ethane, propane and *n*-butane in this shale is interpreted as an indication that only the uppermost portion of a depletion zone was penetrated, that is, the depth was not reached at which this shale has kept its original concentration for these relatively mobile hydrocarbons. The relative abundance and type of distribution of the analysed *n*-alkanes support this conclusion. A distribution envelope of continuously increasing concentrations with increasing carbon number is the opposite of what is known for a high-mature type III kerogen shale like this Pliensbachian shale². Diffusion rates seem to be strongly controlled by molecular size, that is, the shale is depleted in ethane, propane and butane to a much greater depth than for *n*-hexane and *n*-heptane.

Finally, light hydrocarbon concentrations of samples from depths of 7.3 and 7.4 m, shown in Fig. 2, deviate significantly from those of adjacent samples above and below. Both samples tend to be enriched in ethane, propane and *n*-butane, whereas they are depleted in the longer-chain *n*-alkanes. This is interpreted as reflecting the proximity of these samples to an open fracture which was visible in the core at a depth of 7.3 m. This fracture is probably in contact with the atmosphere either directly or through an interconnected fracture system. Downdip and at greater depth, ethane, propane and butane predominantly diffuse from the adjacent shale into the fracture. This hydrocarbon mixture moves upwards along the fracture, probably by a mechanism other than pure diffusion. From this gas-charged fracture ethane, propane and butane have diffused into the immediately adjacent portion of the shale causing the enrichment. The hydrocarbon mixture in the fracture lacks *n*-pentane, *n*-hexane and *n*-heptane compared with the adjacent portions of the shale. Hence those hydrocarbons diffuse towards the fracture resulting in a narrow shale interval parallel to the fracture, which is locally depleted in hexane and heptane.

Quantitative determination of diffusion coefficients

The above observations led us to calculate the absolute values of diffusion coefficients using Fick's law,

$$D \frac{\partial^2 C(z, t)}{\partial z^2} = \frac{\partial C(z, t)}{\partial t} \quad (1)$$

where $C(z, t)$ = concentration of hydrocarbons as a function of depth and time, ng hydrocarbon per g of rock; D = diffusion coefficient, $\text{cm}^2 \text{s}^{-1}$; t = time, s; z = depth, cm.

We assume that the shale unit has, at greater depth, a constant concentration, C_0 , as the initial condition

$$C = C_0 \quad \text{at} \quad \infty \geq z > 0 \quad t = 0$$

The concentration is zero at the outcrop surface, and at an infinite depth the concentration is equal to C_0 for the boundary conditions:

$$C = 0 \quad \text{at} \quad z = 0, \quad t \geq 0$$

$$C = C_0 \quad \text{at} \quad z \rightarrow \infty, \quad t \geq 0$$

Solving the diffusion equation with the above initial and boundary conditions⁸,

$$C(z, t) = C_0 \operatorname{erf} \frac{z}{2(Dt)^{1/2}} \quad (2)$$

The initial concentrations, C_0 , are determined for each *n*-alkane by calculating arithmetic or geometric means of the measured concentrations below the above defined near-surface depletion zones. Concentration values are calculated at 25-cm intervals. $D \cdot t$ is the only unknown required in the calculation of $C(z, t)$ which should correspond with the measured concentration. In mathematical terms, we try to solve an inverse problem by which one can determine the correct parameter of a system. Two techniques were used to optimise $D \cdot t$ —the least-square method, and the comparison of the areas formed between the straight lines joining the measured concentration values and the curve calculated with a certain $D \cdot t$ value. The best value of $D \cdot t$ is found with iteration that minimised the error between

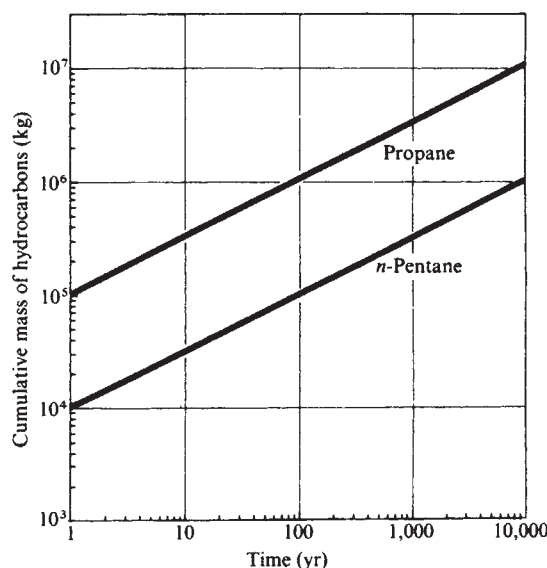


Fig. 4 Calculated cumulative mass of propane and *n*-pentane escaping as a function of time into the atmosphere from a 100 km² outcrop surface of Campanian/Maastrichtian shale on Nûgssuaq Peninsula, West Greenland.

the calculated and measured concentrations. The concentration curve found solving equation (1) is convex.

Using this model, the variation of the light hydrocarbon concentrations with depth could be calculated only for core holes B and E from West Greenland. In both cases the initial concentration C_0 can be determined with reasonable accuracy. The data of core hole H are not suitable for modelling because the diffusion front moved below the depth which was reached by the drill. Once the optimum value of $D \cdot t$ is determined, the absolute value of D is calculated by dividing $D \cdot t$ by the time available for diffusion. The initiation of diffusion for light hydrocarbons is defined here as the time when the present surface of the Earth was established at the sampling locations in the course of uplift and erosion. Although there are no direct means to measure this time exactly, the special geological and geographical conditions of West Greenland enable one to make a reasonably accurate estimate. Present topography results from isostatic uplifting and subsequent erosion following retreat of the thick ice cap which once covered coastal West Greenland. This retreat is dated at 6,000 yr BP (ref. 9), which represents a maximum value for time t to solve equation (2).

As core holes B and E were drilled into shales of the same age and the concentration-depth trends were similar, only the model results for core hole E are discussed here in detail. The concentration curves are calculated from equation (2) with optimised $D \cdot t$ values as described above and plotted together with the measured concentrations for two selected n -alkanes (Fig. 3). The similarity between the convex shape of the calculated concentration curve and the general trend of the measured concentrations strongly supports our observations that the concentration depth-trends of the n -alkanes studied is indeed mainly governed by diffusion.

It was possible to calculate $D \cdot t$ values for n - and iso-butaness and -pentanes, n -hexane and n -heptane in core hole B, and for ethane, propane, n - and iso-butaness and -pentanes in core hole E. Diffusion coefficients calculated (Table 1), vary from 10^{-6} to $10^{-8} \text{ cm}^2 \text{ s}^{-1}$. As Table 1 also shows, they compare favourably with data reported for methane although measured in recent sediments¹⁰. The calculated diffusion coefficients in core holes B and E show a strong dependence on molecular size and geometry: they decrease rapidly with increasing carbon number, except for ethane. The branched alkanes have higher diffusion coefficients than their normal homologues. Comparison of the calculated absolute diffusion coefficients for n - and iso-butaness and -pentanes in core holes B and E shows a good match (for example, for the normal alkanes 3.5×10^{-7} and $1.4 \times 10^{-7} \text{ cm}^2 \text{ s}^{-1}$ in B compared with 3.2×10^{-7} and $1.1 \times 10^{-7} \text{ cm}^2 \text{ s}^{-1}$ in E) which also indicates that the model represents a good first approximation.

Geochemical significance

Based on these absolute diffusion rates several geological processes involving hydrocarbon diffusion can now be quantified. The contribution of diffusion to primary migration of hydrocarbons, thought to involve a combination of different transport mechanisms¹¹, can now be estimated. Within the shale source beds hydrocarbons diffuse over short distances along concentration gradients towards the nearest fracture, vug or silt layer¹. Geochemical surface prospecting is based on the concept of light hydrocarbons diffusing from reservoir accumulations in the subsurface towards the Earth's surface¹². However, a reliable geochemical interpretation of such data should take into account the compositional fractionation effects documented here. Details of these geological applications will be published elsewhere.

The rate at which petrogenic hydrocarbons escape by continuous slow degassing from the lithosphere is of great interest when establishing the global carbon cycle in general and the atmospheric carbon balance in particular¹³⁻¹⁵. Based on the diffusion coefficients, the cumulative mass of propane and n -pentane escaping into the atmosphere as a function of time from

a 100-km² outcrop surface of the Campanian/Maastrichtian shale in West Greenland is calculated and shown in Fig. 4. During the initial periods, as the shale is uplifted and exposed to the surface of the Earth, high amounts of hydrocarbons enter the atmosphere per unit time, (for example, 1.1×10^5 and 1.0×10^4 kg in the first year for propane and n -pentane respectively), decreasing exponentially with time. However, this calculation is based on the simplified assumption that the exposure surface remains constant with time, that is that there is no continued advance of the diffusion front with progressing erosion.

Conclusions

Rocks from the near-surface intervals of three shallow core holes drilled in source bed-type shales exhibit a pronounced depletion in concentration of certain light hydrocarbons (C_2 - C_7) towards the outcrop surface. A major difference is observed in the type and depth of this depletion zone between the three cases examined. In two shale outcrops in West Greenland a depletion zone can only be recognised for ethane through pentane-range hydrocarbons and to a depth of 3 m. In North-west Germany the shale is depleted in ethane to butane-range hydrocarbons to an unknown depth which is >10 m. However, for pentane to heptane-range hydrocarbons the depletion zone extends only to a depth of 5 m. This difference between West Greenland and North-west Germany reflects the influence of permafrost, that is, ice plugging the pore space retards depletion processes.

These near-surface depletion zones are explained as a result of molecular diffusion of light hydrocarbons through the water saturated pore space of the shales along concentration gradients towards the Earth surface. However, several physical factors, which have probably influenced the upward diffusion of light hydrocarbons through the shale section studied, have been neglected in the present stage of our model: adsorption of light hydrocarbons on mineral surfaces, or the inhomogeneity of the porous media, that is subtle changes with depth in clay mineralogy, porosity and permeability.

The concentration-depth trends computed by the mathematical model based on Fick's law closely match those measured for both core holes in West Greenland. An assumption of time available for diffusion in these cases makes it possible to determine absolute diffusion rates of hydrocarbons through a column of rocks in natural conditions. These diffusion coefficients vary between 10^{-6} and $10^{-8} \text{ cm}^2 \text{ s}^{-1}$ for ethane to heptane-range hydrocarbons and decrease sharply with molecular size.

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A DNA cloning system for interspecies gene transfer in antibiotic-producing *Streptomyces*

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Selectable plasmid vectors that contain a gene encoding resistance to the antibiotic methylenomycin A and that are suitable for the cloning of endonuclease-generated DNA fragments in *Streptomyces* species have been constructed and characterised.

STREPTOMYCES are Gram-positive mycelial bacteria which undergo a complex process of morphological differentiation. Because more than 60% of known antibiotics are produced by *Streptomyces* species, including many products of widespread use in human and veterinary medicine and in agriculture, these organisms hold a position of considerable medical and biological importance¹. The development of a DNA cloning system for *Streptomyces* would greatly facilitate the detailed genetic analysis of their antibiotic production pathways and of the molecular mechanisms involved in their differentiation. Furthermore, DNA cloning could produce new combinations of DNA sequences from genetically diverse organisms and perhaps result in new types and increased yields of antibiotics synthesised by these actinomycetes.

Although the process of gene exchange mediated by conjugation within actinomycetes is apparently widespread², the transfer of genetic material between individuals by such sexual means is predominantly restricted to members of the same or closely related species. The recent development of a method for protoplast fusion in *Streptomyces*^{3,4} represents a significant advance in the ability to use genetic recombination as a practical tool in the field of strain improvement; however, the production of recombinants in fused protoplasts by 'homologous recombination' is limited by the extent of DNA sequence similarity. DNA cloning methods potentially permit the insertion of a DNA sequence of any origin into a particular host strain, provided that a suitable vector system is available to propagate the cloned DNA and a means of introducing the recombinant DNA molecule into the host organism exists.

An earlier report⁵ has described a plasmid transformation system that results in the high frequency uptake of covalently closed circular (CCC) DNA by *Streptomyces* in the presence of polyethylene glycol, and allows the visual detection of transformants, after protoplast regeneration, that occur at a frequency of less than 10^{-9} . In optimal conditions, at least 20% of the regenerated protoplasts of *Streptomyces coelicolor* or *Streptomyces parvulus* were transformed with the *S. coelicolor*

plasmid SCP2* and 10^6 transformants per μg of plasmid DNA were obtained; similar results were obtained with *Streptomyces lividans* protoplasts transformed with SLP1 plasmid DNA.

The availability of an efficient system for introducing plasmid DNA into *Streptomyces* species provides a basic tool for DNA cloning in this genus. We report here the development of a *Streptomyces* cloning system using derivatives of two unrelated plasmids, SCP2* and SLP1.2.

Plasmids used for construction of cloning vectors in *Streptomyces*

Two groups of plasmids were potentially suitable for the construction of cloning vectors for *Streptomyces*:

(1) SCP(2) and its high fertility variant, SCP2*, were first isolated from *S. coelicolor* A3(2), and both plasmids have been subjected to genetic and physical analyses⁶⁻⁸. Strains containing either of the plasmids elicit a phenomenon termed lethal zygosis when grown in contact with an SCP2⁻ culture of the same species^{6,8}. Lethal zygosis is associated with plasmid transfer and results in the retardation of the development of the SCP2⁻ culture. This phenotype proved useful in the development of a plasmid transformation system for *Streptomyces* and has facilitated the detection of low-frequency interspecific transfer of SCP2* from *S. coelicolor* to *S. lividans* and *S. parvulus*. Other *Streptomyces* plasmids have subsequently been shown to elicit and confer resistance to lethal zygosis, including SCP1 from *S. coelicolor* and the SLP1 series of plasmids from *S. lividans*. This property seems to be a fairly common phenotype among *Streptomyces* plasmids and may prove useful in identifying additional plasmids that are otherwise phenotypically cryptic. SCP2* and SCP2 have a molecular length of 31 kilobases, are present in the mycelium at about one copy per genome and have identical restriction endonuclease cleavage maps (Fig. 1) for all enzymes used.

(2) The SLP1 series of plasmids was isolated from *S. lividans*⁹. Six different plasmid species SLP1.1-6, of molecular length

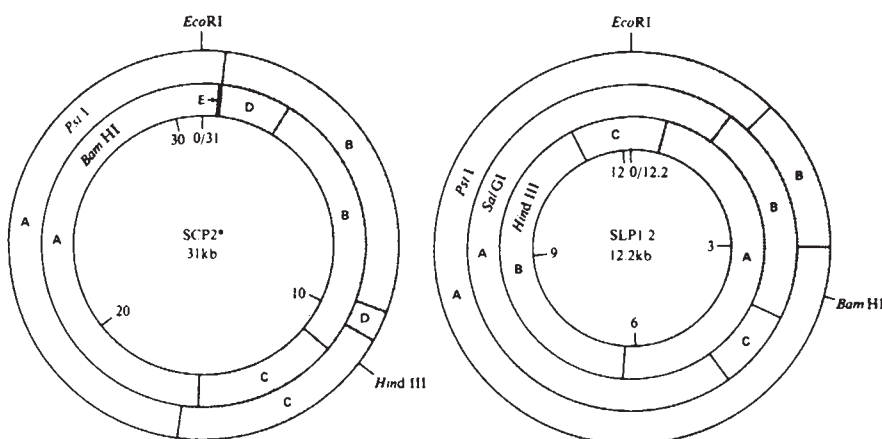
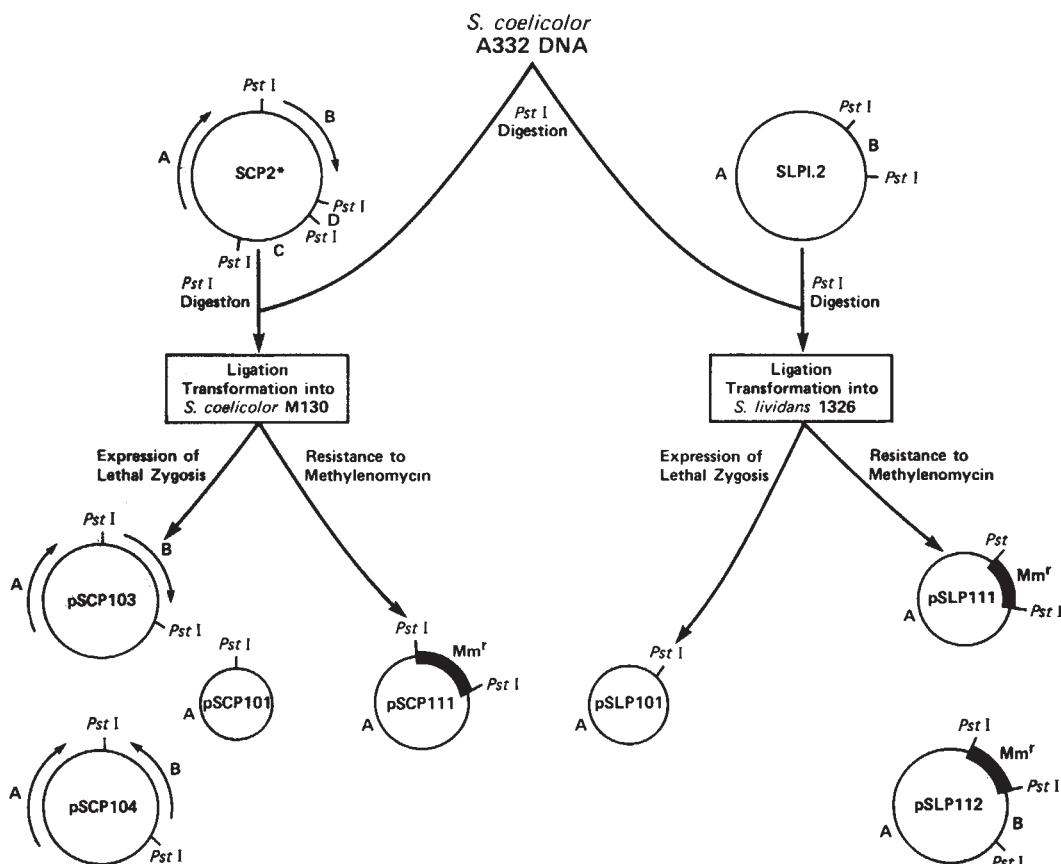


Fig. 1 Restriction endonuclease cleavage maps of the SCP2* and SLP1.2 plasmids. Distances are shown in kilobase (kb) pairs. The maps were derived from single, double and partial digests of SCP2* or SLP1.2 with the enzymes indicated. The shaded areas of each map indicate regions of the genomes which are non-essential for plasmid replication, and which include certain restriction endonuclease cleavage sites that are available for DNA insertion.

Fig. 2 Schematic outlines of the procedure used for cloning the Mm resistance gene(s) or SCP2* and SLP1.2 derived vectors. 0.5 µg of SCP2* DNA, isolated as covalently closed circular (CCC) DNA from *S. coelicolor* strain M109 and 2.5 µg of DNA isolated from *S. coelicolor* strain A332 were digested to completion with *Pst*I, mixed and ligated together at a total DNA concentration of 12 µg ml⁻¹. Similarly, 0.1 µg of SLP1.2, prepared in an identical fashion to SCP2* but from *S. lividans* M180, and 1 µg of A332 DNA were completely digested with *Pst*I, mixed and ligated together at a final concentration of 15 µg ml⁻¹. Each mixture was assayed by agarose gel electrophoresis, precipitated with ethanol, resuspended in 20 µl 10 mM Tris-HCl, pH 8, and 1 mM EDTA (TE buffer) and introduced by transformation into either 10⁷ *S. coelicolor* M130 or 2 × 10⁷ *S. lividans* 1326 protoplasts, respectively. As a control for the spontaneous occurrence of Mm^r-resistant isolates, equal numbers of protoplasts were treated with 20 µl TE buffer in an identical manner. After allowing time for protoplast regeneration and sporulation to occur⁵, spores were collected from the regeneration plates by the addition of 10 ml of sterile distilled water and gentle scraping with a wire loop, filtered through cotton wool, concentrated by centrifugation at 1,000 g for 15 min and resuspended in 0.3 ml 20% glycerol. The spore preparations were then assayed either for the presence of Mm^r clones (Fig. 3) or for expression of lethal zygosis. The remainder of the spore preparation was stored frozen at -20 °C with only slight loss of viability.



9.38–12.35 kilobases, have been isolated and shown by restriction enzyme cleavage mapping to be identical except for a single segment of the plasmid genome. As the size of the plasmid in the series increases, the length of the DNA segment comprising the variable region increases in one direction only from a fixed point. Some of the larger variants consequently acquire additional restriction endonuclease cleavage sites that are contained in DNA sequences not essential for autonomous replication. Thus, insertion of exogenous DNA fragments into these cleavage sites should not interfere with propagation of the plasmid. Particularly attractive as a potential cloning vector is SLP1.2 (12.35 kilobases), which contains a single *Bam*HI and two *Pst*I restriction sites, all within the non-essential variable region (Fig. 1). The SLP1 series of plasmids are self-transmissible and have an estimated copy number of four or five per genome (M.B., unpublished).

Only covalently closed supercoiled plasmid DNA molecules have been used in earlier transformation experiments in *Streptomyces*⁵. Preliminary experiments were therefore carried out to determine the efficiency of uptake of linear and re-ligated plasmid DNA by protoplasts in transformation conditions. SCP2* and SLP1.2 DNA were cleaved into full-length fragments with *Hind*III and *Bam*HI endonucleases, respectively, and subjected to electrophoresis on a 1% agarose gel in Tris-acetate buffer. DNA bands containing linear plasmid DNA molecules were cut out from the gel, separating these from uncleaved molecules present in the endonuclease-treated samples. Linear SCP2* DNA was purified by potassium iodide gradient centrifugation¹⁰, and the linear SLP1.2 fragment was removed from the gel slice by electroelution as described below. Treatment of linear plasmid DNA with bacteriophage T4 ligase resulted in 50% conversion to circles, as estimated by gel electrophoresis (data not shown). Ligated or unligated samples of SCP2* and SLP1.2 were then used for transformation of protoplasts of *S. coelicolor* strain M124 and *S. lividans* strain

1326, respectively, and transformants were identified by their ability to elicit lethal zygosis when placed onto a lawn of a plasmid-minus strain of the same species. Transformation frequencies in excess of 10⁵ per µg DNA were obtained for ligated DNA samples; unligated DNA gave a 10–100-fold lower transformation frequency.

Cloning of a DNA fragment determining resistance to methylenomycin A

Because neither the SCP2* nor the SLP1.2 plasmid contains a known drug-resistance gene readily usable in selection of transformants, we chose in our initial experiments to clone the gene(s) specifying resistance to the antibiotic methylenomycin A (Mm)¹¹; this would then be useful for selection purposes in subsequent cloning experiments. Methylenomycin A has antibiotic activity against a wide range of Gram-positive eubacteria and many *Streptomyces* species. Genes encoding Mm production as well as Mm resistance are present on a genetically but not physically identified plasmid, SCP1 (ref. 12). *S. coelicolor* strain A332 contains the SCP1 plasmid integrated into the chromosome and is believed to possess a single copy of the Mm resistance gene(s) per genome¹³.

The total DNA of *S. coelicolor* A332 was digested with *Pst*I endonuclease, ligated to similarly cleaved SCP2* or SLP1.2 plasmid DNA, and introduced by transformation into *S. coelicolor* or *S. lividans* protoplasts as described in Fig. 2. Spores of each strain were then tested for resistance to Mm and the ability to elicit lethal zygosis. The antibiotic produced by an SCP1-containing strain was used to select for any Mm^r clones present in a lawn of the collected transformation mixtures¹⁴.

Figure 3A illustrates the selection of Mm^r clones of *S. coelicolor*. Mm^r clones of *S. lividans* were selected in a similar way using colonies of *S. lividans* SCP1⁺ strain M233 as a source of Mm. Colonies of *S. coelicolor* and *S. lividans* picked from within

the areas of inhibition of the background lawn were tested for Mm resistance and shown to be resistant to the antibiotic, in contrast to the inhibition observed for strains M130 and 1326 added, respectively, as controls to each of the plates (Fig. 3B).

When purified *S. coelicolor* Mm^r clones were replica-plated to lawns of M130 (*hisA1*, *uraA1*, *strA1*, SCP2⁻) and M110 (*hisA1*, *uraA1*, *strA1*, SCP2^{*}) on supplemented R2 medium plates³, each antibiotic-resistant clone elicited lethal zygosis against M130 but not against M110, indicating the presence of an SCP2^{*}-related plasmid. Similarly, the Mm^r clones of *S. lividans* also exhibited lethal zygosis characteristic of the SLP1 series of plasmids, again indicating the presence of an SLP1 derivative in these clones.

Analysis of methylenomycin-resistant plasmids

Plasmid DNA obtained from Mm^r clones of both *S. coelicolor* and *S. lividans* was isolated using caesium chloride-ethidium bromide density gradient centrifugation⁶. Gel analysis of restriction endonuclease-cleaved CCC plasmid DNA obtained from a randomly selected antibiotic-resistant clone of *S. coelicolor* indicated that it consisted of a single molecular species 18 kilobases long and contained single *Bam*HI and *Eco*RI sites. Further analysis of this plasmid species (designated pSCP111) by endonuclease digestion and gel electrophoresis (Fig. 4) indicated that it contains the largest *Pst*I fragment of SCP2^{*} plus a novel fragment of 2.55 kilobases that presumably carries the gene(s) conferring resistance to Mm.

Similar gel analysis of the endonuclease-cleaved DNA obtained from a Mm-resistant clone of *S. lividans* revealed a plasmid species of molecular length 13.3 kilobases that also includes single cleavage sites for *Eco*RI and *Bam*HI. Additional gel analysis of this plasmid DNA species, designated pSLP111 (Fig. 4), indicated that it consists of the larger *Pst*I fragment of SLP1.2 plus a novel fragment of molecular length 2.55 kilobases. Another independently isolated Mm^r-resistant clone of *S. lividans* yielded a full-length SLP1.2-derived plasmid (pSLP112) that contained a 2.55 kilobase *Pst*I-generated fragment inserted into one of its two *Pst*I sites (Fig. 2). Hence, three independently obtained Mm^r clones all contained plasmid derivatives that included a *Pst*I-generated DNA fragment 2.55 kilobases long, suggesting that this inserted fragment included the Mm resistance gene(s). In each case the cloned *Pst*I-generated fragments lacked sites for *Bam*HI, *Eco*RI and *Hind*III.

Table 1 Stability of SCP2^{*} derivatives

Plasmid	% Loss per life cycle	
	In <i>S. coelicolor</i> strain M130	In <i>S. lividans</i> strain 1326
SCP2 [*]	0.5 (2/420)	4 (9/224)
pSCP101	9.0 (15/168)	35 (79/224)
pSCP102	7.0 (12/168)	34 (95/280)
pSCP103	0.3 (0/336)	NT
pSCP104	0.5 (0/232)	NT
pSCP111	22.0 (50/224)	NT
	35.0 (90/254)*	NT

For all derivatives, spores were scraped from single colonies that expressed lethal zygosis when replicated onto a lawn of an SCP2⁻ strain, serially diluted and plated onto complete medium. The resulting colonies were assayed for their ability to express lethal zygosis by replication onto lawns of SCP2⁻ strains of the same species. In addition, for pSCP111, a portion of the spores from a single colony was streaked at 90° to a culture of M146 on complete medium to test for Mm resistance; this test confirmed the presence of pSCP111 in the spore that gave rise to the assayed colony. Spores from the original colony were suspended in water, serially diluted and plated onto complete medium. The resulting single colonies were then streaked against M146 to assay again for Mm resistance, which showed the continued presence of pSCP111. These plates were later replicated to lawns of M130 on supplemented R2 medium to assay for the ability of the isolates to express lethal zygosis. All Mm^r isolates expressed lethal zygosis and all Mm^r isolates did not, indicating that the loss of pSCP111 rather than DNA rearrangements resulted in a Mm-sensitive phenotype. Numbers in parentheses indicate the number of colonies not showing lethal zygosis (or for pSCP111 not showing Mm resistance) over the total number of colonies. NT, not tested.

* Loss of Mm resistance.

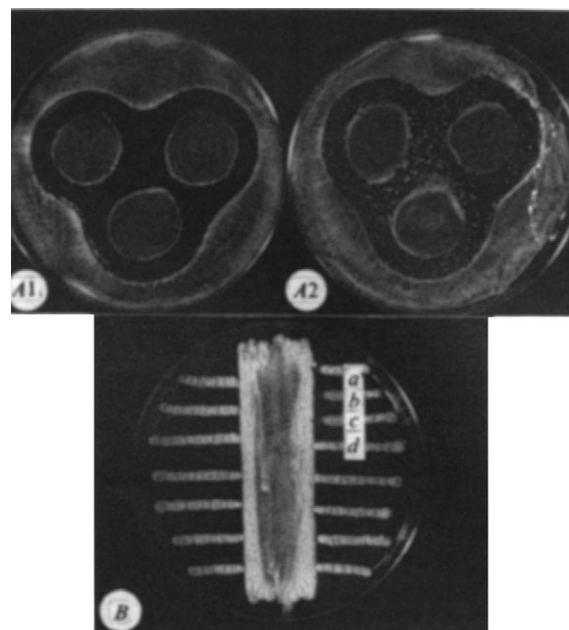


Fig. 3 Selection of Mm^r clones. **A**, 0.1-ml volumes of the collected *S. coelicolor* M130 transformation and control mixtures were spread onto complete medium agar²³ and the plates allowed to dry. Three patches of *S. coelicolor* SCP1⁺ strain M146 were applied to both plates, which were then incubated at 30 °C for 4–6 days: (1) transformation mixture resulting from the treatment of M130 protoplasts with TE buffer; (2) transformation mixture resulting from the treatment of M130 protoplasts with a ligated mixture of *Pst*I-cleaved SCP2^{*} and A332 total DNA. The presence of resistant colonies in the area of inhibition of the lawn produced by diffusion of Mm from the M146 patches is seen. **B**, Colonies growing in the zones of inhibition of plate A2 were streaked at right angles to a culture of the SCP1⁺ strain M146 on complete medium and incubated at 30 °C for 3–5 days. Aerial mycelium formation and sporulation of the Mm-sensitive control cultures M130 (SCP1⁻, SCP2⁻) (**a**), M110 (SCP1⁻, SCP2^{*}) (**b**) and 1190 (SCP1⁻, SCP2^{*}) (**c**) are inhibited proximal to the antibiotic produced by M146. Streaks of the clones picked from A2 and of strain M146 (**d**) were resistant to Mm.

To confirm that the novel fragment conferring Mm resistance did, in fact, contain the Mm^r gene(s) from the chromosomally integrated SCP1 plasmid, a labelled probe of pSCP111 was made by nick translation¹⁵ using [α -³²P]dCTP. This probe was then hybridised using the method of Southern¹⁶ (with modifications) to *Pst*I-cut total DNA that was isolated from two SCP1⁺ and two SCP1⁻ isolates of *S. coelicolor* (Fig. 5). The labelled pSCP111 DNA hybridised to only the *Pst*I-A fragment of SCP2^{*} (15.4 kilobases) and to DNA fragments of 2.55 kilobases present in the SCP1 containing strains A332 and M146. No hybridisation to DNA fragments of this size could be seen with the SCP1⁻ M130 and M110 DNA samples.

Interspecific expression of pSCP111

The ability to transfer genes among different *Streptomyces* species and to obtain phenotypic expression in the new host is of potential importance in the use of DNA cloning methods for the development of new antibiotics. To investigate whether the cloning system described above is likely to be of general use in *Streptomyces* species, we transformed derivatives of *S. lividans* and *S. parvulus* with pSCP111 and selected for Mm resistance using the method described previously. Protoplasts were prepared from cultures of *S. lividans* 1326 and *S. parvulus* 2283 and transformed with 10 ng of purified pSCP111 DNA. After protoplast regeneration, the resulting spore preparations were assayed for the presence of Mm clones using *S. lividans* strain M233 (SCP1⁺) and *S. coelicolor* strain M146 (SCP1⁺), respectively, as sources of Mm for the *S. lividans* and *S. parvulus* transformation mixtures. In both cases antibiotic-resistant colonies were obtained. Plasmid DNA was isolated from the *S. lividans* and *S. parvulus* Mm^r-resistant derivatives using caesium chloride-ethidium bromide density gradient centri-

Table 2 Fertility properties of SCP2* derivatives

Components of mating	Colony-forming units per ml on ^a :				Average recombination frequency [†]	Plasmid transfer frequency (% per recipient)
	HUS (Parentals)	PAC	PACUS (Recombinants)	PCH		
M130 × M124	3.5 × 10 ⁹	1.2 × 10 ⁹	1.2 × 10 ²	20	3.9 × 10 ⁻⁸	—
M130 (SCP2*) × M124	5.0 × 10 ⁸	5.6 × 10 ⁸	2.1 × 10 ⁵	3.7 × 10 ⁶	3.7 × 10 ⁻³	100 (100/100)
M130 (pSCP101) × M124	6.4 × 10 ⁷	2.8 × 10 ⁸	1.4 × 10 ⁴	2.2 × 10 ⁵	1.2 × 10 ⁻³	95 (106/112)
M130 (pSCP102) × M124	1.3 × 10 ⁸	8.0 × 10 ⁷	1.2 × 10 ⁴	2.1 × 10 ⁵	1.1 × 10 ⁻³	93 (104/112)
M130 (pSCP103) × M124	1.9 × 10 ⁸	4.4 × 10 ⁸	4.2 × 10 ⁵	8.2 × 10 ⁵	2.3 × 10 ⁻³	100 (112/112)
M130 (pSCP104) × M124	2.6 × 10 ⁹	2.3 × 10 ⁹	4.2 × 10 ⁶	1.2 × 10 ⁷	3.3 × 10 ⁻³	100 (112/112)
M130 (pSCP111) × M124	3.5 × 10 ⁸	8.3 × 10 ⁸	3.5 × 10 ⁵	9.9 × 10 ⁵	1.35 × 10 ⁻³	78 (87/112) 55 (74/134)‡

Matings were carried out as previously described²³. Plasmid transfer frequency was determined by patching colonies from the PAC plates on to minimal medium containing proline, arginine and cysteine and after sporulation replicating to a lawn of M130 spread on fully supplemented R2 medium. The colonies were then scored for their ability to elicit lethal zygosis.

^a Minimal medium containing some of the following supplements: histidine (H), uracil (U), streptomycin (S), proline (P), arginine (A) and cysteine (C).

[†] Expressed as the average of the mean recombination frequencies of each parent.

[‡] The transfer frequency of pSCP111 was also determined by streaking colonies from the PAC plates at right angles to M146 on complete medium and scoring for Mm resistance. M130 = *S. coelicolor* hisA1, uraA1, strA1, SCP1⁻, SCP2⁻; M124 = *S. coelicolor* proA1, argA1, cysD18, SCP1⁻, SCP2⁻.

fugation and was indistinguishable from pSCP111 by gel analysis of *Pst*I endonuclease digests.

We also transferred pSCP111 from *S. coelicolor* to *S. lividans* and *S. parvulus* by mating, selecting for Mm^r exconjugants. We have demonstrated, therefore, that the pSCP111 vector can be introduced by transformation or conjugation into two different *Streptomyces* species, *S. lividans* and *S. parvulus*, and that transformants can in each instance be selected by expression of the Mm^r gene(s) cloned in the autonomously replicating pSCP111.

Plasmid derivatives of SCP2* and SLP1.2 and their properties

SCP2* is an autonomously replicating extrachromosomal element and so must contain at least one origin of replication. The plasmid is self-transmissible and presumably includes functions that accomplish its inter-bacterial transfer among different strains of *S. coelicolor*, although the precise mechanism of conjugation in these mycelial organisms is unknown. SCP2* expresses functions that determine the level of chromosomal recombination between two genetically distinguishable derivatives of *S. coelicolor* by a mechanism that is possibly related to the transfer properties of the plasmid. It also confers on its host cell production of and resistance to lethal zygosis⁶. The lethal zygosis phenotype depends on plasmid transfer, and resistance to lethal zygosis may be functionally analogous to the surface exclusion phenomenon expressed by the sex factor F in *Escherichia coli*¹⁷.

To localise the basic biological functions of the SCP2* plasmid, derivatives of SCP2* that contain only particular restriction fragments of the original plasmid were isolated. The *Pst*I-digested and ligated *S. coelicolor* transformation mixture used originally to select for pSCP111 (Fig. 2) was also diluted and plated on supplemented R2 medium to which sufficient spores of a culture of M130 had been added to produce a confluent lawn. After incubation at 30 °C for 3–4 days, growth-centred zones of inhibition of the background lawn characteristic of lethal zygosis were observed. Spores were picked from the centre of these zones of inhibition and streaked out to obtain single colonies which, on replication to lawns of M130 on supplemented R2, expressed lethal zygosis. In a similar manner, SCP2* digested with *Bam*HI endonuclease was ligated and introduced into M130, and single colonies which expressed lethal zygosis on lawns of M130 were purified.

Isolation of CCC DNA from these clones and subsequent restriction enzyme analysis using *Bam*HI and *Pst*I single and double digests confirmed unambiguously that the isolates all contained derivatives of SCP2* having the structures indicated

in Fig. 2. pSCP101 (15.4 kilobases) consisted of the *Pst*I-A fragment of SCP2*; pSCP102 (15.9 kilobases) consisted of the *Bam*HI-A fragment of SCP2* (not shown); pSCP103 (24.3 kilobases) consisted of the *Pst*I-A+B fragments of SCP2* orientated as in the original plasmid; and pSCP104 (24.3 kilobases) consisted of the *Pst*I-A+B fragments of SCP2* but in the opposite orientation.

As both pSCP101 and pSCP102 replicate autonomously, an origin of replication is necessarily located in the *Pst*I-A and *Bam*HI-A fragments of the parent plasmids. Similarly, as all these plasmids express lethal zygosis when replicated on to an SCP2⁻ lawn and also confer resistance to this phenotype when used as lawns themselves, the gene(s) involved in production of and resistance to lethal zygosis must also be located in this region.

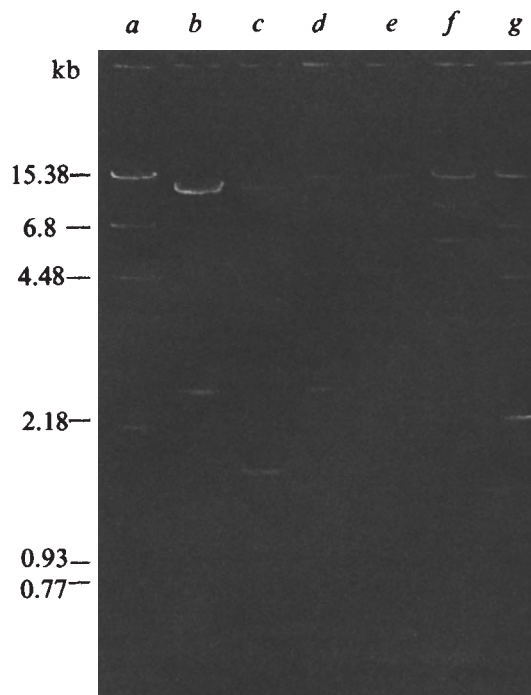


Fig. 4 Gel analysis of restriction endonuclease-cleaved pSCP111 and pSLP111 plasmid derivatives. Lanes a and g, SCP2* DNA digested with *Bam*HI and *Pst*I generating fragments of 15.4, 6.8, 4.3, 2.18, 0.93 and 0.8 kilobases. All other plasmids digested with *Pst*I. b, pSLP111; c, SLP1.2; d, pSCP111; e, pSCP101 (see below); f, SCP2*. Electrophoresis was from top to bottom in 1% agarose in 40 mM Tris-acetate, pH 7.9, and 1 mM EDTA for 4 h at 50 mA.

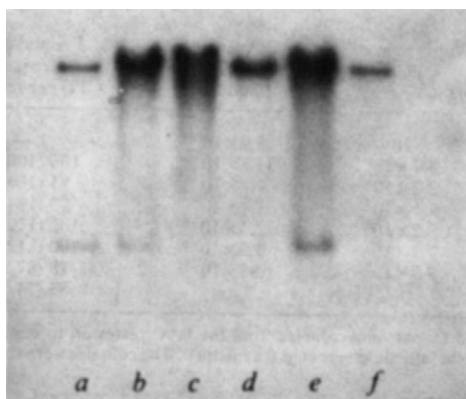


Fig. 5 Hybridisation of ^{32}P -labelled pSCP111 DNA to isolated DNA from *S. coelicolor* Mm-resistant or sensitive isolates. Aliquots (5 μg) of DNA isolated from each of the *S. coelicolor* strains A332 (chromosomally integrated SCP1, SCP2*), M146 (SCP1⁺, SCP2⁻), M110 (SCP1⁻, SCP2*) and M130 (SCP1⁻, SCP2⁻) were digested with 1.5 U *Pst*I at 37 °C for 3 h and electrophoresed as indicated in Fig. 4. Samples of SCP2* and pSCP111 DNA digested with *Pst*I were also included as controls. The gel was stained in 0.5 $\mu\text{g ml}^{-1}$ ethidium bromide, photographed and then soaked twice in 250 ml 0.25 M HCl for 15 min to depurinate the DNA and so enhance transfer of the larger fragments from the gel to the nitrocellulose filter²⁴. The gel was rinsed with water and soaked twice in 250 ml 0.5 M NaOH and 1 M NaCl for 15 min to denature the DNA. The gel was washed briefly three times with 200 ml water and then neutralised by soaking it for 20 min in 200 ml 3 M NaCl and 0.5 M Tris-HCl, pH 7.5. DNA was then transferred from the gel to the nitrocellulose filter soaked with 20 $\times$ SSC, pH 6.5 (1 $\times$ SSC = 0.3 M NaCl, 0.03 M sodium citrate); after 5 h of contact between filter and gel, no DNA could be detected in the gel on staining with ethidium bromide. The nitrocellulose filter was baked at 80 °C in a vacuum oven for 2 h and then prehybridised at 52 °C in the presence of 50% formamide, 6 $\times$ SSC, pH 6.5, 25 mM sodium phosphate, pH 6.5, 0.1% sodium pyrophosphate, 10 $\mu\text{g ml}^{-1}$ boiled calf thymus DNA and 1% glycine for 12 h. 0.5 μg pSCP111 DNA was incubated at 15 °C for 2.5 h in the presence of 100 μCi [α - ^{32}P]dCTP (12.5 μM), 12.5 μM each of dATP, dGTP, dTTP, 1 $\mu\text{g ml}^{-1}$ pretreated DNase and 0.1 U μl^{-1} DNA polymerase (Boehringer-Mannheim) in 50 mM Tris-HCl, pH 7.8, 5 mM MgCl₂, 50 mg ml⁻¹ bovine serum albumin and 10 mM 2-mercaptoethanol in a total volume of 20 μl . The mixture was extracted with phenol and passed over a Sephadex G-50 fine column in 20 mM Tris-HCl, pH 8, 50 mM NaCl and 1 mM EDTA to remove unincorporated label. The resulting probe had a specific activity of 1.8×10^8 c.p.m. per μg . The prehybridised nitrocellulose filter was then hybridised to 10⁷ c.p.m. of the radioactively labelled pSCP111 probe, previously denatured by heating to 108 °C for 20 min in a saturated NaCl bath. Hybridisation conditions were similar to prehybridisation conditions except that 5 $\times$ SSC was used and calf thymus DNA and glycine were absent. Incubation was at 52 °C for 24 h in a total volume of 1.2 ml in a sealed polyethylene bag. The filter was then rinsed twice with 50 ml of hybridisation solution, incubated three times in 50 ml of the same solution at 52 °C for 1 h and washed twice for 1 h at 52 °C in hybridisation solution containing 0.5 $\times$ SSC. The filter was finally rinsed with 2 $\times$ SSC, dried and exposed to X-ray film for 12 h. The lanes contain: a, pSCP111; b, M146 DNA (SCP1⁺, SCP2⁻); c, M130 DNA (SCP1⁻, SCP2⁻); d, M110 DNA (SCP1⁻, SCP2*); e, A332 DNA (chromosomally integrated SCP1, SCP2*); f, SCP2*.

The stability of each of the SCP2* derivatives was determined in *S. coelicolor* (Table 1). The stabilities of SCP2*, pSCP101 and pSCP102 were also examined in *S. lividans* after introduction of the plasmids into this species by either mating (SCP2*) or transformation (pSCP101 and pSCP102). As shown in Table 1, the pSCP111, pSCP101 and pSCP102 derivatives were significantly more unstable than the parental SCP2* plasmid in both *S. coelicolor* and *S. lividans*. However, in *S. coelicolor*, addition of the *Pst*I-B fragment of SCP2* in either orientation to the plasmid derivative totally restored stability to the level shown by SCP2*. These observations are analogous to those made by P. A. Meacock and S.N.C. for *E. coli* plasmids¹⁸; they have isolated a region of pSCP101 that accomplishes the active partitioning of plasmid molecules in dividing cells. We suggest that the *Pst*I-B fragment of the *Streptomyces* plasmid SCP2* may also carry information for partitioning of the plasmid between dividing cells. However, definitive studies of the segregation of plasmids are difficult in a mycelial organism which

may contain several chromosomal genomes per cell and which undergoes a complex process of differentiation and sporulation.

The transfer properties of the plasmid vectors and their abilities to promote chromosomal recombination were also studied. Examination of the fertility properties of each of the SCP2* derivatives (Table 2) indicates that SCP2* increases the level of chromosomal recombination approximately 10⁵-fold over that observed in the absence of any plasmids and that this plasmid is transferred at a frequency of 100% to the recipient culture⁶. Each of the derivatives of this plasmid had fertility and transfer properties similar to those of the parent, indicating that the fertility determinants of SCP2* as well as the transfer functions are located in the region of the plasmid genome covered by the *Pst*I-A and *Bam*HI-A fragments.

In experiments analogous to those described above, isolates were obtained from the *S. lividans* transformation mixture (Fig. 2) that elicited lethal zygosis against *S. lividans* 1326. Plasmid DNA extracted from these clones was analysed by digestion with *Pst*I or *Eco*RI endonucleases and shown to consist of the larger *Pst*I fragment of SLP1.2. This derivative, designated pSLP101, contains unique *Pst*I and *Bam*HI sites located in a non-essential region of the plasmid; one of the sites for both *Hind*III and *Sal*I also occurs in this region. This plasmid is potentially useful for the cloning of DNA fragments produced by any of these four enzymes.

Conclusions

The findings reported here demonstrate the practicality of DNA cloning and heterospecific expression of cloned genes in *Streptomyces*. Because transformation of *Streptomyces* protoplasts is highly efficient, it is possible to clone genes from the chromosome of donor DNA preparations. One of the vectors we have constructed (pSCP111) replicates and expresses resistance to methylenomycin in at least three different *Streptomyces* species, each of which produces at least one antibiotic (refs 11, 19, 20 and M.B., unpublished). Each vector contains an antibiotic resistance determinant that is directly and easily selectable in most *Streptomyces* species and has single cleavage sites in non-essential regions for several different restriction endonucleases, some of which yield DNA termini especially useful for cloning a wide variety of differently generated fragments. For example, DNA fragments generated by *Bgl*II, *Bcl*I, *Sau*3A or *Mbo*II have protruding 5' ends identical to those produced by *Bam*HI and can be cloned at the *Bam*HI site of these vectors. In addition, fragments of chromosomal DNA produced by random shearing can be introduced into the *Pst*I cleavage site by a dG-dC homopolymeric tailing procedure (for example, see ref. 21).

The ability to clone genes in *Streptomyces* should prove valuable in the analysis and, ultimately, the manipulation of antibiotic synthetic pathways of these organisms²². Such manipulation may enable new antibiotics to be produced by the introduction of exogenously derived enzymes that modify pre-existing antibiotics or lead to the synthesis of hybrid antibiotic molecules. In addition, the cloning on multi-copy plasmids, such as pSLP101, of genes involved in rate-limiting biosynthetic steps may prove useful in increasing the yield of antibiotics produced by *Streptomyces* species.

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²H NMR study of molecular motion in collagen fibrils

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Collagen was labelled through tissue culture with [3,3,3-d₃]alanine. ²H NMR spectra were obtained of the labelled collagen as fibrils and in solution using the quadrupolar echo technique for solids. The ²H NMR data were analysed in terms of a model for reorientation in which the molecule is considered to jump between two orientations in a time which is short compared to the residence time in each site, and short compared to (Δν_Q)⁻¹. The best fit of the data indicates that the collagen molecule in the fibrils experiences reorientation about its long axis over an angular range of ~30–40°. The T₂ for [3,3,3-d₃]alanine-labelled collagen fibrils is estimated to be ~110 μs.

CURRENT information about the spatial distribution of amino acids in the collagen molecule has mainly come from X-ray fibre diffraction and model building^{1–4}. These methods, by their nature, lead to a concept of a static collagen molecule in the fibril. We report here ²H NMR results which, in conjunction with previous ¹³C NMR results^{5,6}, require a motionally dynamic model for the collagen molecule in the fibril. We have used solid state ²H NMR of [3,3,3-d₃]alanine-labelled collagen to obtain information about molecular motion of the collagen peptide backbone in the fibrils, and to obtain preliminary information on the orientation of the C^α–C^β bond axis of alanine residues in the collagen molecule. In addition, we present equations which allow a simple calculation of the ²H NMR lineshape in the presence of motion. These results provide strong evidence that the peptide backbone in collagen fibrils undergoes rapid (τ_c ~ 10⁻⁷ s) motion about the long axis of the molecule and that this motion takes place over an ~30–40° range in azimuthal angle. To our knowledge, this work represents the first application of solid state ²H NMR as a probe for the molecular dynamics of a labelled protein, although this technique has been used very successfully in the membrane field⁷.

Solid state ²H NMR is well suited for the study of molecular motion for several reasons. First, relaxation in ²H NMR is dominated by the quadrupolar interaction, and therefore no questions arise concerning the source of the relaxation mechanism in ²H NMR. Second, the deuterium field gradient tensor for most C–D bonds is generally axially symmetric with the unique principal axis along the C–D bond direction⁸, which makes spectral interpretation straightforward. Third, ²H NMR quadrupolar powder patterns are sensitive to motions which have correlation times τ ≈ (Δν_Q)⁻¹, where Δν_Q is the deuterium quadrupole splitting⁷. Fourth, the low natural abundance (0.016%) of deuterium virtually eliminates background signals due to unlabelled material in natural abundance.

Labelling of collagen and measurement of spectra

Lathyrus collagen was labelled with [3,3,3-d₃]alanine through chick calvaria culture by previously described methods^{5,6}. Characterisation of the protein by amino acid analysis indicated that it was pure collagen⁹. Optical rotatory dispersion at 313 nm (ref. 10) established that the entire collagen sample was in native form on completion of the ²H NMR experiments. The per cent

incorporation of deuterated alanine in the protein was determined by chemical ionisation gas chromatography–mass spectroscopy of *N*-acetyl methyl ester derivatives^{5,6} of the enzymatically hydrolysed protein. A small amount of proton exchange occurs at alanine during acidic hydrolysis of proteins¹¹. An enzymatic hydrolysis procedure (Pronase and aminopeptidase M) was therefore developed for collagen to avoid artefacts introduced by acid-catalysed exchange on the determination of the per cent incorporation of ²H. Radiotracer analysis (both ³H and ¹⁴C) indicated that no amino acids other than alanine were labelled during biosynthesis. The labelled collagen was studied by ²H NMR both in solution (~10 mg ml⁻¹ in 0.1 M acetic acid) and as fibrils (in equilibrium with 0.02 M Na₂HPO₄). ²H NMR spectra were obtained using a 'home-built' spectrometer operating at 5.2 T (33.78 MHz for ²H). The π/2 pulse was 5 μs. A solid echo pulse sequence (90°_x – t₁ – 90°_y – t₂ – T) (refs 12–14) was used for all experiments to preserve the inhomogeneously broadened portion of the NMR signal, which is normally lost through spectrometer dead time. The phase of the first 90° pulse was shifted by 180° on alternate scans to reduce artefacts due to coil ringing and imperfect 90° pulses. All data were acquired in quadrature (2,000 points per channel) with a sampling rate of 5 μs per point. Signal distortion due to pulse power fall-off was less than 10% over the range ±40 kHz, and the lineshapes presented here were not corrected. A control ²H NMR spectrum of unlabelled collagen was run for every ²H NMR spectrum of labelled collagen discussed here. This ensured that the portion of the signal arising from [3,3,3-d₃]alanine-labelled collagen and the portion arising from deuterated water in natural abundance could be assigned with confidence.

Analysis of the spectra

The ²H NMR spectra for L-[3,3,3-d₃]alanine and for various alanine-labelled collagen samples are presented in Fig. 1. As a powdered amino acid, L-[3,3,3-d₃]alanine exhibits the classical ²H NMR powder pattern (Fig. 1a). The observed quadrupolar splitting (Δν_Q = (3e²qQ/4h)(3cos²θ' – 1)/2) of 38.8 kHz (Table 1) shows that the static quadrupolar coupling constant (e²qQ/h ≈ 165 kHz) has been averaged to approximately one-third of its value by rapid rotation of the methyl group¹⁵. Frozen [3,3,3-d₃]alanine-labelled collagen fibrils (Fig. 1b) exhibit a ²H NMR lineshape similar to that of the powdered amino acid. The

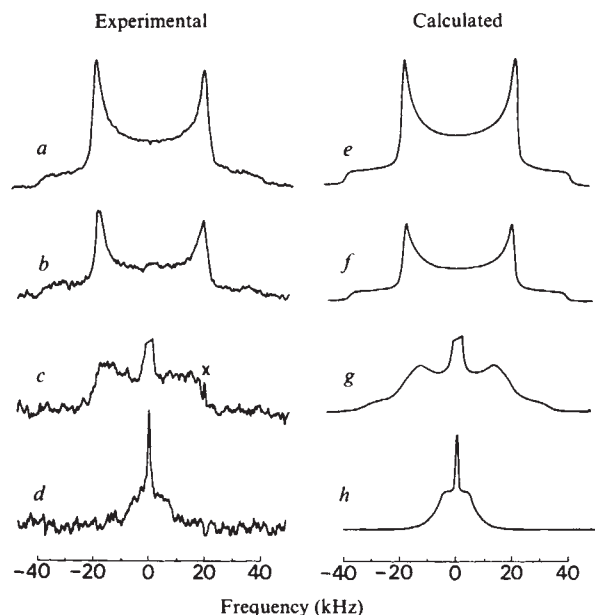


Fig. 1 Experimental ^2H NMR spectra for $[3,3,3\text{-d}_3]\text{alanine}$ and $[3,3,3\text{-d}_3]\text{alanine}$ -labelled collagen. These spectra were obtained in quadrature using the solid echo pulse sequence ($90^\circ_{\text{xx}} - t_1 - 90^\circ_{\text{yy}} - t_2 - T$) with t_1 and t_2 set at 50 and 59 μs , respectively. *a*, $[3,3,3\text{-d}_3]\text{Alanine}$ as a polycrystalline amino acid; 256 accumulations, 0.25 s repetition rate, 18 $^\circ\text{C}$; *b*, $[3,3,3\text{-d}_3]\text{alanine}$ -labelled collagen fibrils, 20 mg in equilibrium with excess Na_2HPO_4 ; 2×10^5 accumulations, 0.25 s repetition rate, -18 $^\circ\text{C}$; *c*, the same as in *b*, except at 18 $^\circ\text{C}$; *d*, $[3,3,3\text{-d}_3]\text{alanine}$ -labelled collagen in solution, ~ 10 mg/ml in about 0.4 ml 0.1 M acetic acid in deuterium-depleted water; 2.5×10^5 accumulations, 0.1 s repetition rate, 18 $^\circ\text{C}$; *e-h*: calculated spectra for $[3,3,3\text{-d}_3]\text{alanine}$ and for $[3,3,3\text{-d}_3]\text{alanine}$ -labelled collagen. Lorentzian line-broadening was used in all cases; *e*, calculated spectrum of polycrystalline $[3,3,3\text{-d}_3]\text{alanine}$; $\Delta\nu_q = 38.8$ kHz, line-broadening = 1.3 kHz; *f*, Calculated spectrum of frozen $[3,3,3\text{-d}_3]\text{alanine}$ -labelled collagen fibrils; $\Delta\nu_q = 37.3$ kHz, line-broadening = 1.5 kHz; *g*, Calculated spectrum of $[3,3,3\text{-d}_3]\text{alanine}$ -labelled collagen fibrils at 18 $^\circ\text{C}$. The alanine $\text{C}^\alpha\text{-C}^\beta$ bond axes are assumed to undergo jump diffusion between two equally populated sites. The angles θ between the $\text{C}^\alpha\text{-C}^\beta$ bond axes and the helix axis are 69° and 85° (Fig. 2*a, b*). The angle 2δ through which the projected alanyl $\text{C}^\alpha\text{-C}^\beta$ bond axis is carried by reorientation of the peptide backbone is 40° (Fig. 2*b, c*). The spectra calculated for $\theta = 69^\circ$ and $\theta = 85^\circ$ were summed with equal weight; $\Delta\nu_q = 37.3$ kHz, line-broadening = 4.1 kHz. A Lorentzian signal with 600 Hz linewidth was included in the centre of the spectrum to simulate the natural abundance signal from water. *h*, Calculated spectrum of $[3,3,3\text{-d}_3]\text{alanine}$ -labelled collagen in solution. Reorientation of the alanyl $\text{C}^\alpha\text{-C}^\beta$ bond axis about the long axis of the helix has averaged the crystalline 37.3 kHz quadrupolar splitting to ~ 10 kHz. Line-broadening = 4.1 kHz.

quadrupolar splitting for the frozen fibrils is about 3% smaller than for the labelled amino acid (Table 1). This result may arise from a small amount of molecular motion (in addition to methyl rotation) which occurs in the fibrils at -18 $^\circ\text{C}$, or may reflect a slightly smaller quadrupolar coupling constant for alanine in a polypeptide.

At +18 $^\circ\text{C}$, $[3,3,3\text{-d}_3]\text{alanine}$ -labelled collagen fibrils exhibit a ^2H NMR spectrum in which about 60% of the signal intensity is lost and in which the singularities are no longer well defined (Fig. 1*c*; Table 1). To establish that the observed lineshape (Fig. 1*c*) was representative of the entire sample, a spectrum was obtained at the shortest values of t_1 and t_2 which were consistent with recovery of the spectrometer (30 and 39 μs , respectively). This spectrum contained more intensity, but was identical in shape (within uncertainty due to baseline distortion) to the spectra obtained at longer t_1 and t_2 values. The apparent reduction of the quadrupolar splitting and the loss of signal intensity

through homogeneous T_2 processes¹⁶ observed in Fig. 1*c* imply reorientation of the alanine methyl in collagen fibrils in addition to 3-fold rotation. Motion of the alanine methyl group (the $\text{C}^\alpha\text{-C}^\beta$ bond axis) requires reorientation of the peptide backbone in collagen because alanine contains no additional bonds about which reorientation can take place.

The ^2H NMR spectrum of $[3,3,3\text{-d}_3]\text{alanine}$ -labelled collagen in solution shows a further reduction of the spectral width (Fig. 1*d*; Table 1). In solution, collagen behaves as a rod-like monomer of molecular weight 285,000 and dimensions of $\sim 1.5 \times 300$ nm¹⁷. The molecule in solution can undergo free rotation about its long axis, ($R_1 \sim 10^7$ s⁻¹), whereas end-over-end rotation is slow ($R_2 \sim 10^2$ s⁻¹) and is restricted at these concentrations due to occupied volume considerations^{5,6}. Accordingly, the ~ 37 -kHz quadrupolar splitting observed for the frozen labelled fibrils would be expected to collapse by a factor of $(1 - 3\cos^2\theta)/2$ in the presence of free rotation about the long axis of the collagen molecule. Here, θ is the angle formed between the $\text{C}^\alpha\text{-C}^\beta$ bond axis and the long axis of the molecule (Fig. 2*a*). The observed quadrupolar splitting for labelled collagen in solution is ~ 10 kHz, which requires that θ be $\sim 70^\circ$. An angle of $\sim 70^\circ$ for θ is in good agreement with the $\text{C}^\alpha\text{-C}^\beta$ bond angles predicted by various models for the collagen helix^{1,3,18} which place θ between 60° and 90° . Comparison of the ^2H NMR intensity from the labelled sample with the intensity from the same amount of unlabelled collagen (deuterium-depleted water used in both cases) indicates that about half the intensity of the sharp peak in Fig. 1*d* arises from the alanine-labelled protein in solution and that the rest is due to deuterated water in natural abundance. Previous ^{13}C NMR experiments^{5,6},

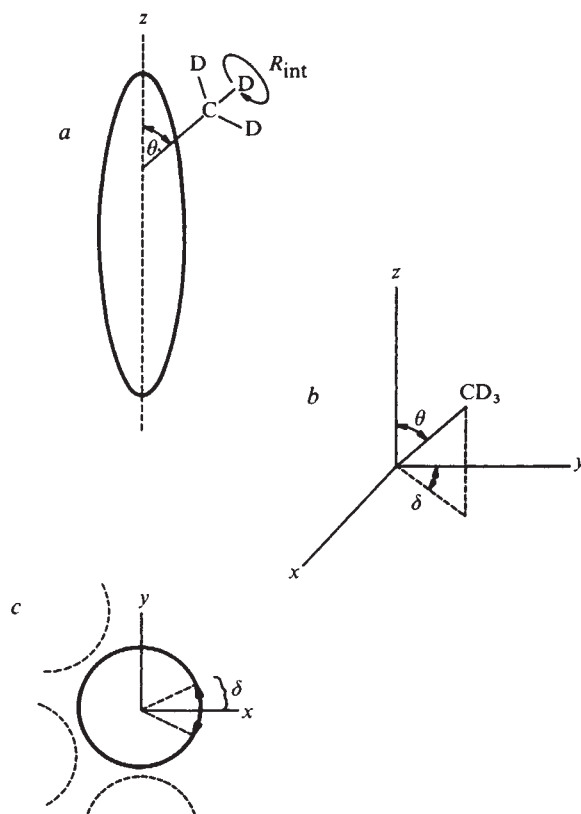


Fig. 2 Schematic representation of the alanine methyl group attached to the collagen helix. *a*, The collagen helix considered as an ellipsoid. The $\text{C}^\alpha\text{-C}^\beta$ bond of alanine makes the angle θ with the long axis of the helix; *b*, the methyl group of alanine, which makes an angle θ with respect to the helix axis (the z axis), undergoes reorientation between two sites separated by an angle 2δ ; *c*, Cross-section of collagen molecules assembled into fibrils. The molecules undergo reorientation about the long axis of the helix over an azimuthal angle of 2δ .

Table 1 Quadrupolar splitting ($\Delta\nu_q$) for [3,3,3- d_3]alanine and [3,3,3- d_3]alanine-labelled collagen fibrils

Sample	$\Delta\nu_q^*$ (kHz)
[3,3,3- d_3]Alanine	38.8
[3,3,3- d_3]Alanine-labelled collagen fibrils in 0.02 M Na_2HPO_4	
-18°C	37.3
+18°C	~30†
lyophilised	38
[3,3,3- d_3]Alanine-labelled collagen in solution	~10†

* $\Delta\nu_q = \frac{3e^2qQ}{4h} (3 \cos^2 \theta' - 1)/2$; θ' is the angle made by the $\text{C}^\alpha\text{--D}$ and $\text{C}^\alpha\text{--C}^\beta$ bond axes.

†Axially asymmetric pattern; value reported is the frequency difference between maxima in the calculated spectrum and corresponds to the apparent splitting.

in conjunction with the present sample characterisation, indicate that it is unlikely that this peak is due to a small amount of denatured protein. However, the sharp component may be due to some alanine $\text{C}^\alpha\text{--C}^\beta$ bond axes which make the 'magic angle' (54.7°) with respect to the helix axis, or to the 3% of the alanine residues which reside in the non-helical termini of the molecule².

The ^2H NMR spectra of labelled collagen fibrils were analysed by matching the experimental lineshapes with lineshapes calculated in the presence of molecular reorientation. In the motional model utilised for these calculations, the molecule is considered to jump between two orientations in a time which is much less than the residence time in each of the two sites¹⁹ and much less than $(\Delta\nu_q)^{-1}$.

The assumed motion of the collagen backbone makes the alanine $\text{C}^\alpha\text{--C}^\beta$ bond axis jump between two orientations, labelled 1 and 2 in Fig. 3a. As a consequence of the motion, the observed powder pattern will not generally be axially symmetric. Using Fig. 3a, one can determine the principal axis system (x, y, z) and the frequencies ($\omega_x, \omega_y, \omega_z$) corresponding to the principal values of the motionally averaged field gradient tensor as follows. For clarity we consider the powder pattern corresponding to the transition for which $\omega_\perp \leq \omega \leq \omega_\parallel$ (see Fig. 3b). When H_0 (the applied field) is normal to the 1, 2 plane, $\omega = \omega_\perp$ in both the 1 and 2 orientations. Since this is the minimum value possible for ω , x must be normal to the 1, 2 plane (we use the convention $\omega_x \leq \omega_y \leq \omega_z$) and $\omega_x = \omega_\perp$. The remaining principal axes, y and z , are perpendicular to x and are thus in the 1, 2 plane. When H_0 is in this plane:

$$\begin{aligned}\omega &= 0.5[\omega_\parallel(\cos^2 \theta_{1H} + \cos^2 \theta_{2H}) \\ &\quad + \omega_\perp(\sin^2 \theta_{1H} + \sin^2 \theta_{2H})] \\ \omega &= -0.5\omega_\perp[3 \cos^2 \theta_{1H} + 3 \cos^2(\gamma_{12} - \theta_{1H}) - 2]\end{aligned}$$

where θ_{1H} and θ_{2H} are the respective angles made by H_0 with 1 and 2. When H_0 lies along z , ω is maximal, and solving the equation $d\omega/d\theta_{1H} = 0$ shows that z bisects γ_{12} when γ_{12} is in the first or fourth quadrants, and that z is perpendicular to the bisector of γ_{12} when $90^\circ \leq \gamma_{12} \leq 270^\circ$. The y axis is normal to the xz plane. The frequencies corresponding to the field along the principal axes are

$$\begin{aligned}\omega_x &= \omega_\perp \\ \omega_y &= -\omega_\perp[3 \sin^2(\gamma_{12}/2) - 1] \\ \omega_z &= -\omega_\perp[3 \cos^2(\gamma_{12}/2) - 1]\end{aligned}$$

These results apply for γ_{12} in the first or fourth quadrant. When $90^\circ \leq \gamma_{12} \leq 270^\circ$, the expressions for ω_y and ω_z are interchanged. Note that an axially symmetric pattern occurs only when $\gamma_{12} = 0, 90$ or 270° (Fig. 3b), and a pattern having $\eta = 1$ occurs whenever ω_y equals zero (Fig. 3c). The correspondence

between γ_{12} and the angles (θ, δ) which describe the reorientation of the molecule is: $\cos \gamma_{12} = \cos^2 \theta + \sin^2 \theta \cos^2 2\delta$.

The relevant angles which describe the orientation of the alanine methyl group in the collagen molecule are shown in Fig. 2. The angle formed by the alanine $\text{C}^\alpha\text{--C}^\beta$ bond axis and the long axis of the collagen helix is designated as θ (Fig. 2a,b). The angle δ is half of the angle through which the alanine $\text{C}^\alpha\text{--C}^\beta$ bond is carried by reorientation of the peptide backbone (Fig. 2b,c).

The various collagen models predict that the angle which the $\text{C}^\alpha\text{--C}^\beta$ bond makes with the helix axis depends on whether the considered amino acid occurs in the X or Y position of the Gly-X-Y triplet sequence^{1,3,18}. Accordingly, the lineshapes from two representative values of θ (85° and 69°) were summed with equal weight (alanine is distributed equally in the X and Y positions in collagen) for each calculated spectrum. The angle δ and the line broadening were varied until the best match of the lineshape was obtained.

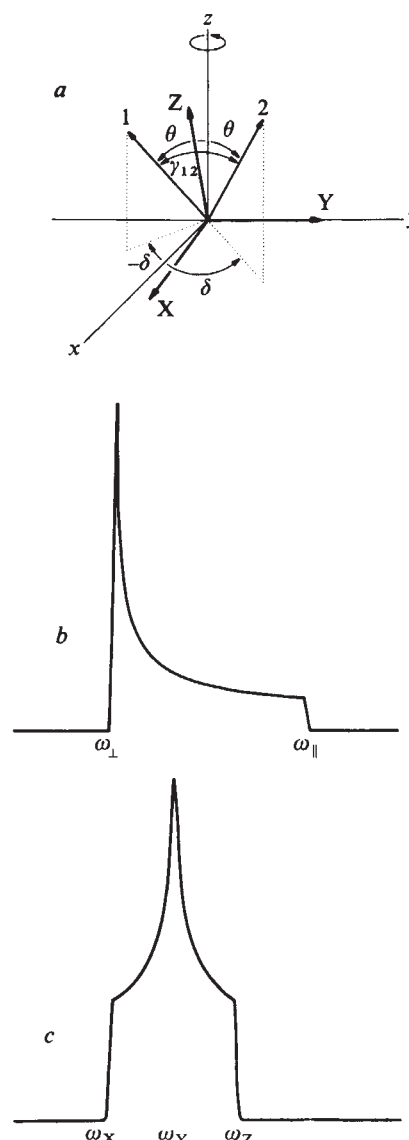


Fig. 3 a, The geometrical construction used to deduce ω_x, ω_y and ω_z in the presence of two-site jump reorientation. 1 and 2 refer to the two orientations between which the alanine $\text{C}^\alpha\text{--C}^\beta$ bond axis is considered to jump. The angles θ, δ , and γ_{12} are described in the text; b, a theoretical axially symmetric powder pattern (one transition for the case when $\eta = 0$); c, the theoretical pattern expected when $\eta = 1$ (when $\omega_y = 0$). In the presence of line-broadening, this pattern would lose definition in the edges and have an overall tent-like appearance.

Implications for models of collagen

The best fit of the data (Fig. 1c,g) indicates that the collagen molecule in the fibril undergoes reorientation over a ~ 30 – 40° range in azimuthal angle (that is, $2\delta \approx 30$ – 40° ; Fig. 2c). This angle is in good agreement with an estimate obtained from previous ^{13}C NMR studies,^{5,20} in which the minimum angle, 2δ , consistent with the ^{13}C T_1 data, was $\sim 30^\circ$. The assumption used in the calculations of the ^2H NMR lineshapes (that the jump rate is much larger than $\Delta\nu_q$) is consistent with the reorientation rate of about 10^7 s^{-1} , derived from the ^{13}C studies^{5,6,20}. A line-broadening of $\sim 4\text{ kHz}$ is required to fit these ^2H NMR data. A portion of this line-broadening ($\sim 3\text{ kHz}$) can be explained by the T_2 , which we estimate to be $\sim 110\text{ }\mu\text{s}$. The rest of the line-broadening is attributed to D–H dipolar interactions. Mass spectroscopic analysis showed that of the alanine residues which were labelled, about half contained three deuterons, one quarter

contained two deuterons, and one quarter contained one deuteron. Note that the partial loss of deuterons from alanine must occur during biosynthesis as mass spectroscopy indicates that $\sim 95\%$ of the starting $[3,3,3\text{-d}_3]\text{alanine}$ and $\sim 95\%$ of the control labelled alanine for the enzymatic hydrolysis contained three deuterons. A second moment calculation⁸ indicates that the static D–H interaction between a deuteron and a single proton on the same alanine methyl carbon²¹ causes an average line-broadening of $\sim 3.5\text{ kHz}$. Three-fold rotation of the methyl group would average this to $\sim 1.7\text{ kHz}$.

The ^2H NMR results presented here, in conjunction with earlier ^{13}C NMR results^{5,6,20}, provide strong evidence that the peptide backbone in collagen fibrils undergoes rapid ($\tau_c \leq 10^{-7}\text{ s}$) anisotropic motion about the long axis of the molecule. The range in azimuthal angle over which this motion takes place is estimated to be 30 – 40° .

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LETTERS

The γ -ray background and the age of protogalaxy formation

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The diffuse γ -ray background which is implied by the current models of protogalaxy formation is considered here in an attempt to set limits to the redshift z_i of that event. This is obtained by observing that cosmic rays from supernovae exploding in protogalaxies produce an isotropic γ -background in excess of the observed values if protogalaxies form too early. Without any special assumptions, the constraints on z_i turn out to be more significant than any other limit previously reported. In fact, for z_i to be >100 , one must postulate either a very low cosmic ray production in supernovae, or a very low density of intergalactic gas, that is, $<10^{-10}\text{ cm}^{-3}$, or both.

We consider only specific cases which set the age of galaxy formation at a redshift $z_i \leq 100$. In this case, even for a 'dense interstellar gas universe' ($n_0 \approx 10^{-5}\text{ particles cm}^{-3}$), all proton and photon absorption and degradation effects become minimal, and we can consider the simplified model of γ -ray propagation in a vacuum by thin target scattering.

In contrast, if $z_i \geq 100$, a different model, which can be simplified according to Cavallo and Rees¹, and Cavallo and Pacini² should be used. However, the predictions of this second approach depend critically on the precise shape of the 'fireball' photon spectrum in the neighbourhood of 0.5 MeV, a shape that has not yet been reported. Therefore, we shall only consider the simpler case $z_i \leq 100$, which already gives some interesting results.

The circumstances of protogalaxy formation are well known: at a given z_i , appropriate light element gas clouds collapse and

fragment, producing first generation stars. These stars will evolve according to canonical models, and will manufacture the heavier elements which will then be found in the second generation stars. Supernova explosions will expel and mix these heavier elements in the interstellar gas, and their abundances can presently be observed.

Note that supernovae are also thought to be the source of cosmic ray energy. Cosmic rays in their turn will produce γ -rays through the decay of neutral mesons produced in collisions with the interstellar gas. Current views, therefore, suggest that when protogalaxies formed light gas was converted into heavier elements and cosmic rays. Such a conversion into high energy particles might be approximated by a burst of cosmic rays taking place at a certain time z_b , in general quite close to z_i . In turn, such a burst might be incompatible with the presently observed γ -ray flux. To calculate a minimum value for the γ -ray background, we assume that cosmic rays directly or indirectly receiving their energy from supernova explosions interact mainly with the intergalactic gas. If the galaxies or, worse, the remnants, were opaque to cosmic rays, the energy conversion into γ -rays would be much more efficient, and our limits on z_i would thus be significantly lowered. Similarly, our results would be strengthened if the origin of the γ -background were not due to the mechanisms considered here.

The relevant computations go back to Stecker³ and Cavallo⁴, who obtained substantially similar results. The differential flux observed at $z = 0$ is given by

$$I_\gamma(E_\gamma) = cn_0 H_0^{-1} \int_0^{z_b} G_\gamma(E_\gamma; 1+z) \times (I(z)/I_b)(1+z)^{-5/2} dz \quad \text{photons cm}^{-2} \Delta E^{-1} \quad (1)$$

Here we have assumed that the cosmic ray flux $I(z)$ has the same energy dependence as I_b , the presently observed galactic cosmic ray flux; moreover, an Einstein–de Sitter model has been adopted. The function $G_\gamma(E)$ is the differential specific emissivity produced by collisions of the galactic cosmic ray flux with the

interstellar gas in a 1-cm pathlength. One can show that, for $E_{\gamma e} = E_{\gamma o}(1+z)$ (subscripts e and o refer to emitted and observed respectively) the differential emissivity becomes: $G_g(E; 1+z) = (1+z)G_g[(1+z)E]$. For a burst model, one has³:

$$I(z)/I_g = A(1+z)^3(1+z)^{3/2}(1+z_b)^{-3/2} \quad (2)$$

where one should observe the normalisation $I_o/I_g = A(1+z_b)^{-3/2}$. Thus the integral becomes:

$$I_{\gamma}(E_{\gamma}) = cn_0 H_0^{-1} A(1+z_b)^{-3/2} \int_0^{z_b} G_g[E_{\gamma}(1+z)](1+z)^3 dz \quad (3)$$

An easy comparison with experiment can be made by integrating the flux for $E_{\gamma} \geq 100$ MeV. If $y = E_{\gamma}(1+z)$, we obtain:

$$I_{\gamma} \leq (E_{\gamma} > 100 \text{ MeV}) = cn_0 H_0^{-1} A(1+z_b)^{-3/2} \int_0^{z_b} (1+z)^2 dz \times \int_{100 \text{ MeV}/(1+z)}^{\infty} G_g(y) dy \quad (4)$$

Above 100 MeV and for $z \geq 3$ (a value comparable with the maximum z observed in QSOs) a good approximation is the relationship

$$\int_{(1+z)100 \text{ MeV}}^{\infty} G_g(y) dy = 2 \times 10^{-25} (1+z)^{-3/2} \quad (5)$$

Therefore:

$$I_{\gamma}(E_{\gamma} > 100 \text{ MeV}) = 2 \times 10^{-25} cn_0 H_0^{-1} (I_o/I_g)(2/3)[(1+z_b)^{3/2} - 1] \quad (6a)$$

$$= 1.3 \cdot 10^{-25} cn_0 H_0^{-1} (I_o/I_g)(1+z_b)^{3/2} \quad \text{for } z_b \gg 1 \quad (6b)$$

Equation (6b), divided by 4π , may be compared with the flux 1×10^{-5} photons $(\text{cm}^2 \text{sr})^{-1}$, given in ref. 5, smaller by a factor of 10 than the flux adopted by Stecker³. The desired result is, therefore,

$$I_o/I_g \leq [1 \times 10^{-2}](1+z_b)^{-3/2} h_{100} n_5^{-1} \quad (7)$$

where $h_{100} = H_0/(100 \text{ km s}^{-1} \text{ Mpc}^{-1})$ and $n_5 = n_0/(10^{-5})$. Equation (7) gives a good approximation to the values given in ref. 3, apart from the factor of 10 mentioned above. The theory of star formation gives some indication of the value of I_o/I_g . Our model assumes that a fraction of $\sim 2.8 \times 10^{-6} E_{50}$ protons per supernova of at least $6 M_{\odot}$ goes into cosmic rays. Here, E_{50} is the total energy emitted in cosmic rays of average energy 3 GeV, measured in units of 10^{50} erg. If all matter had gone through the supernova stage, the ratio I_o/I_g would be $\sim E_{50}/4$ at present. In fact, only an appropriate fraction $f(z_b)$ of all matter went into supernovae, specifically, only those stars whose mass exceeds $\sim 6 M_{\odot}$. The general result is therefore:

$$f(z_b)(1+z_b)^{3/2} \leq [1 \times 10^{-2}](E_{50}/4)^{-1} h_{100} n_5^{-1} \quad (8)$$

Here a method of computing $f(z_b)$ is used based on current fragmentation models, which predict a specific minimum mass M_{jt} for the ultimate fragments. M_{jt} depends on the background temperature according to the law⁶:

$$M_{jt} = 1.54 \times 10^{-3} (m_H/m)^2 T_b^2 (k_{pf}/k_0) M_{\odot} \quad (9)$$

with $T_b = 2.7(1+z)K$; m_H and m are the mass of the hydrogen atom and of the typical atom of the fragmenting gas cloud, k_{pf} is the final Planck mean opacity per unit mass, and k_0 is the opacity due to free electron scattering in ionised hydrogen. The existence of such a temperature dependent minimum mass ensures us that the earlier the protogalaxies formed, the more complete was the conversion of light gas into heavier elements and cosmic rays.

Assuming a Salpeter⁷ initial mass function $\psi(m) = K m^{-2.3}$, we can write:

$$f(z_b) = \int_{6M_{\odot}}^{\infty} m\psi(m) dm / \left(\int_{M_H}^{\infty} m\psi(m) dm \right) \quad (10)$$

which becomes:

$$f(z_b) = \begin{cases} 0.15(1+z_b)^{0.6} & \text{for } z_b \leq 22.12 \\ 1 & \text{for } z_b \geq 22.12 \end{cases} \quad (11a)$$

$$(11b)$$

Consequently one should satisfy the relationship:

$$0.27 \geq (1+z_b)^{21/10} E_{50} n_5 h_{100}^{-1} \quad \text{for } z_b \leq 22 \quad (12a)$$

$$4 \times 10^{-2} \geq (1+z_b)^{3/2} E_{50} n_5 h_{100}^{-1} \quad \text{for } z_b \geq 22 \quad (12b)$$

Thus, in general to have $z_b \geq 3$, one needs $\epsilon E_{50} n_5 h_{100}^{-1} \leq 1.5 \times 10^{-2}$ where ϵ is the efficiency of protogalaxy formation. The current value $\epsilon = 1$, and we conclude that in this framework ($n = 10^{-5} \text{ cm}^{-3}$) there is no chance of finding an appropriate z_b . The only effective possibility is that of lowering n_0 to, say, 10^{-7} cm^{-3} . In this case we would obtain:

$$1+z_b \approx 4.8(E_{50} n_7 h_{100}^{-1})^{-10/21} \quad (13)$$

and we suggest this value of $z_b \approx 4$ as a possible burst age. An intergalactic gas density of $< 10^{-10}$ is needed to achieve $z_b \geq 100$. Of course, this does not mean that we favour an open universe: we can only say that the intergalactic gas is likely to give an irrelevant contribution to the average matter density of the universe.

The foregoing results depend on some common assumptions which we now examine.

(1) The validity of equations (9) and (10): In fact, the $(1+z)$ dependence in equation (9) is weakened in equation (10), and would be practically ineffective for a $\psi(m) = K m^{-2}$ power law. Thus, equation (9) does not strongly affect our results. The possibility that the initial mass distribution is appreciably different from the Salpeter function is more important. One must, however, observe that supernovae are needed for manufacturing heavy elements, without producing too many of them. There are two alternatives: (i) the initial distribution of stars, although non-Salpeter-like, mostly consists of massive stars; (ii) the initial distribution mostly consists of low mass stars. The first alternative, which supports our conclusions, has the risk of producing too many heavy elements, but in the second alternative the minimum mass should be such that supernovae can produce a sufficient amount of heavy elements, without leaving behind too many first generation stars. Therefore, the assumption implicit in equation (10) is not really critical.

(2) The validity of the assumption that each supernova produces 10^{50} erg in cosmic rays. At first, this might seem to be a very uncertain figure, as it depends on two poorly known parameters—the explosion rate and the cosmic ray diffusion coefficient. However, estimates of the total particle content for many remnants⁸, and considerations of the kinetic energy input in remnants, using some equipartition assumptions⁹, all indicate a rather restricted range of values around and perhaps greater than $E_{50} = 1$. A finer point is to consider the fate of the protons thus produced. It is commonly assumed that the energy requirements for cosmic rays are met by supernovae: the cosmic rays might directly come from the first stages of the explosion (as in the Colgate¹⁰ model) or the energy of supernovae might be used to accelerate cosmic rays by interstellar shocks. In both cases our results will stand.

(3) Finally, protogalaxy formation might have a small efficiency. Hogan and Layzer¹⁰ made similar assumptions to explain the microwave and X-ray background, but they had to resort to a high yield of relativistic electrons per supernova ($3-7 \times 10^{48}$ erg). The above criticisms also apply to their model, but our results on z_b turn out to be more restrictive, while giving information about the intergalactic gas density which the previous authors could not consider. Our stricter limits, apart from minor technical details, such as the assumption of an Einstein-de

Sitter universe with a slightly higher conversion of particles into relativistic cosmic rays, result from the following: (1) the multiplicity of meson production is >1 and increases with energy; (2) there are about 100 times more protons than electrons in cosmic rays; (3) most important of all, the energy content of the diffuse X-ray background exceeds by about two orders of magnitude the energy content in the isotropic γ -ray background.

Therefore we believe that γ -ray astronomy is still one of the most useful sources of information on the early universe.

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Inhibition of diffusion by the reflection effect in binary Hg–Mn stars

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The Hg–Mn stars constitute a sub-group of the peculiar A stars which have abundance anomalies in their atmospheres. Their distinctive properties are excesses of P, Mn, Ga, Y and Hg¹, slow rotation², a high frequency of double-lined spectroscopic binaries^{3,4}, and a lack of detectable magnetic fields⁵. It is widely believed that the abundance anomalies are at least partly due to the separation of elements by diffusion under the influence of gravitation and radiation pressure^{6,7}. For the diffusion mechanism to operate the atmosphere has to be extremely quiescent with circulation velocities $\leq 10^{-3}$ m s⁻¹. We point out here that in a spectroscopic binary system with similar components surface streaming velocities greatly in excess of this value are expected due to the mutual irradiation of the components.

The mutual irradiation creates temperature gradients over the respective surfaces of the binary components; hydrostatic equilibrium cannot be maintained and streaming away from the substellar points must occur. The mean effective temperature, T , over each illuminated hemisphere is given by

$$T^4 \sim T_0^4 (1+f)$$

T_0 being the effective temperature of the averted hemispheres and f being the fraction of radiation from one star intercepted by the other. For a mean component separation a and a component radius R ,

$$f \sim 1/4(R/a)^2$$

The horizontal temperature gradient produces a horizontal pressure difference ΔP which drives a streaming motion with characteristic velocity v . Thus for a characteristic photospheric density ρ

$$\Delta P \sim \frac{1}{2} \rho v^2$$

which with the gas equation, and in the absence of a significant magnetic field and radiation losses, yields

$$v \sim 0.18 (\Delta T)^{1/2} \text{ km s}^{-1}$$

where

$$\Delta T = T - T_0 \sim T_0 f/4$$

(compare with ref. 8 where a coefficient 0.36 was derived by approximate application of the equation of motion).

The periods of known Hg–Mn spectroscopic binaries range from 3 to 560 days; 13 of the 22 known binaries have periods of less than 10 days, and eight of these are double-lined with mass ratios near unity^{3,4}. For a typical Hg–Mn binary with a period of 6 days and components of masses $\sim 6M_\odot$, $a \sim 2 \times 10^{10}$ m. With $R \sim 2.7 \times 10^9$ m, $f = 4.6 \times 10^{-3}$. Taking $T_0 \sim 14,000$ K, one finds $\Delta T \sim 16$ K and $v \sim 700$ m s⁻¹. Radiative losses are likely to reduce v by a factor of $\sim 10^2$ (ref. 9). Thus the characteristic streaming velocity will be ~ 7 m s⁻¹. This velocity is four orders of magnitude greater than those envisaged on the diffusion mechanism. The Reynolds number is $\geq 10^7$ and the streaming is turbulent. Then the ratio of mixing to diffusive time scales is simply the ratio of the respective velocities, and so the photospheric layers will mix on a time scale $\leq 10^{-4}$ of the segregation time for diffusion in a quiescent atmosphere.

The question arises whether a magnetic field might constrain the motion. Equating magnetic and kinetic energies,

$$H^2/8\pi \sim \frac{1}{2} \rho v^2$$

where H is the magnetic field strength. Hence if $\rho \sim 10^{-9}$ g cm⁻³, $H \sim 0.08$ G. The threshold of detection of the line-of-sight component of H is ~ 200 G, and so a magnetic field might possibly constrain the stream motion. It is not clear whether turbulent motions will occur in an atmosphere dominated by a magnetic field. However, even if laminar flow and diffusion occur over restricted regions of the stellar surface, vertical mixing must still take place at the boundaries of these regions.

Synchronous rotation has been assumed in the foregoing discussion. In this case the streaming pattern would be stable, but mixing at the boundary between the illuminated and averted hemispheres might prevent the formation of a spectrum variable. However, measurements of projected rotational velocities⁴ show that Hg–Mn stars in short-period binaries often have rotational periods longer than the orbital periods. With non-synchronous rotation the illumination will affect the various parts of the stellar surface in turn. The flow pattern is quickly established, and so streaming motion will be periodically induced over the entire stellar surface.

The streaming will decrease very rapidly with the optical depth τ , and diffusion at sub-photospheric layers, say $\tau \geq 5$, is not precluded by the reflection effect. However, models accounting for the overabundances and isotopic anomalies of Hg in terms of diffusive segregation at high levels¹⁰ seem to be excluded; almost all Mn stars have large overabundances of Hg (refs 11, 12).

The abundance anomalies of Hg–Mn stars do not seem to be correlated with their measured rotational velocities¹³. Such a correlation might have been expected if all the anomalies were due to diffusion limited only by meridional circulation.

The only current alternatives to diffusion are accretion models involving supernova ejecta¹⁴ or planetary bodies¹⁵ and so on. Simple accretion may produce overabundances but not deficiencies. However, we have not excluded sub-photospheric diffusion, and this may account for some of the observed deficiencies. Thus the explanation of Hg–Mn star anomalies may lie in a combination of accretion and sub-photospheric diffusion mechanisms.

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Santa Catharina meteorite and phase composition of irradiated Fe–Ni Invar alloys

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The iron meteorite Santa Catharina is an ataxite that contains 35% Ni. Laboratory made Fe–Ni alloys of this composition are called Invar alloys, due to their low thermal expansivity near room temperature. The heterogeneous phase composition of the meteorite, which is apparent from a metallographic examination, has been confirmed by thermomagnetic measurements¹ and by more recent Mössbauer spectra and microprobe analysis². The nature and origin of the alloy phases in Santa Catharina have been the subject of recent investigations^{3,4} and we report here Mössbauer spectra and X-ray diffraction measurements which show that the phase composition of the meteorite is similar to that obtained by electron irradiation of Fe–Ni Invar alloys⁵.

The extensive phase segregation and radiation-induced ordering which occurs in electron irradiated Invar alloys have been observed by small angle neutron scattering⁶ and by Mössbauer spectroscopy⁵. This is illustrated in Fig. 1a, b. One of the phases is paramagnetic and causes the central peak of the spectrum (Fig. 1b), and the other phase is ferromagnetic and causes the magnetic hyperfine splitting of the Mössbauer spectrum. Computer fitting shows that the latter spectrum consists of two six-line spectra which have asymmetric shapes caused by the presence of an electric field gradient which superimposes a quadrupole splitting on the magnetic hyperfine pattern. Both spectra are due to the ordered Fe–Ni phase with the L10 superstructure^{7,8}. One of the two overlapping spectra has an hyperfine magnetic field $H_i = 288$ kG and a quadrupole splitting $\Delta E_Q = +0.23$ mm s⁻¹. This spectrum is caused by large crystallites of the ordered phase having their magnetic axes parallel to the axes of the electric field gradient⁹. Small crystallites may have their magnetic axes perpendicular to the axes of the electric field gradient and thus produce different Mössbauer parameters ($H_i = 310$ kG, $\Delta E_Q = -0.12$ mm s⁻¹).

Figure 1c shows the spectrum obtained with a thin section of the Santa Catharina meteorite showing a well-resolved asymmetric Mössbauer spectrum with a magnetic hyperfine field $H_i = 288$ kG, and quadrupole splitting $\Delta E_Q = +0.20$ mm s⁻¹ and a central peak corresponding to a paramagnetic phase. The Mössbauer parameters corresponding to the magnetic phase shows that this phase in the meteorite is composed only of large crystallites of the ordered alloy with the L10 superstructure, and all their magnetic axes are parallel to the electric field gradient axes.

We have also measured the Mössbauer spectra of the irradiated alloy and of the meteorite in other conditions, such as at low temperatures (4.2 K), with powder absorbers, under the influence of an applied magnetic field up to 50 kG; no obvious differences were observed between the samples.

In Fig. 2, the X-ray diffraction pattern obtained with the unirradiated alloy (35% Ni) is compared with the irradiated alloy and the meteorite sample. Superstructure L10 lines are detected in the irradiated alloy and in the meteorite but with much higher intensity. This corresponds to what was observed in the Mössbauer spectra, that is, that the degree of ordering is much larger in the meteorite compared with that obtained by irradiation of the alloy.

In Fig. 3, the thermal variation of the X-ray lattice parameters of the unirradiated alloy is compared with the irradiated alloy and the meteorite sample.

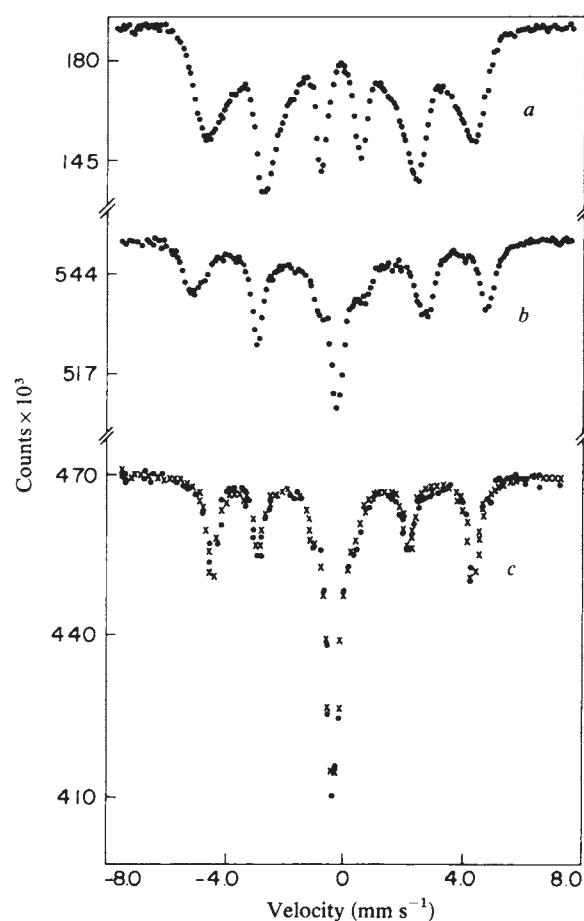


Fig. 1 Mössbauer absorption spectra recorded at room temperature with a ⁵⁷Co source in Rh of: a, Fe–Ni Invar alloy (35% Ni) before irradiation; b, after irradiation at 80 °C with 4×10^{19} 3-MeV electrons; c polished thin section of the Santa Catharina meteorite.

A large decrease of the lattice parameter, from 3.593 Å (Fig. 3a) to 3.582 Å (Fig. 3b) is observed on irradiating the Invar alloy. The two phases which are formed by irradiation have the same value for the lattice parameter. The Invar properties of the alloy are shown to disappear on irradiation by the linear variation of the lattice parameter with temperatures up to 300 °C. The same behaviour is observed with the sample of Santa Catharina, which exhibits a non-Invar thermal behaviour of the lattice parameter (Fig. 3c₁). The Invar property returns when the sample is heated at 800 °C for 24 h in a non-oxidising atmosphere (Fig. 3c₂).



Fig. 2 001 row of the reciprocal space observed with a single crystal diffractometer using the $K\alpha$ Co filtered radiation: a, Fe-Ni Invar alloy before irradiation; b, Fe-Ni Invar alloy after irradiation; c, Santa Catharina meteorite.

These results show that the Fe-Ni phases in Santa Catharina exhibit the same properties of the irradiated Fe-Ni Invar alloy in which phase segregation and radiation-induced ordering occurs with the formation of the L10 superstructure. Basically, the radiation increases the kinetics of the phase segregation and ordering of the alloy. Essentially the same processes occur in the meteorite, but on a much different time scale due to the exceed-

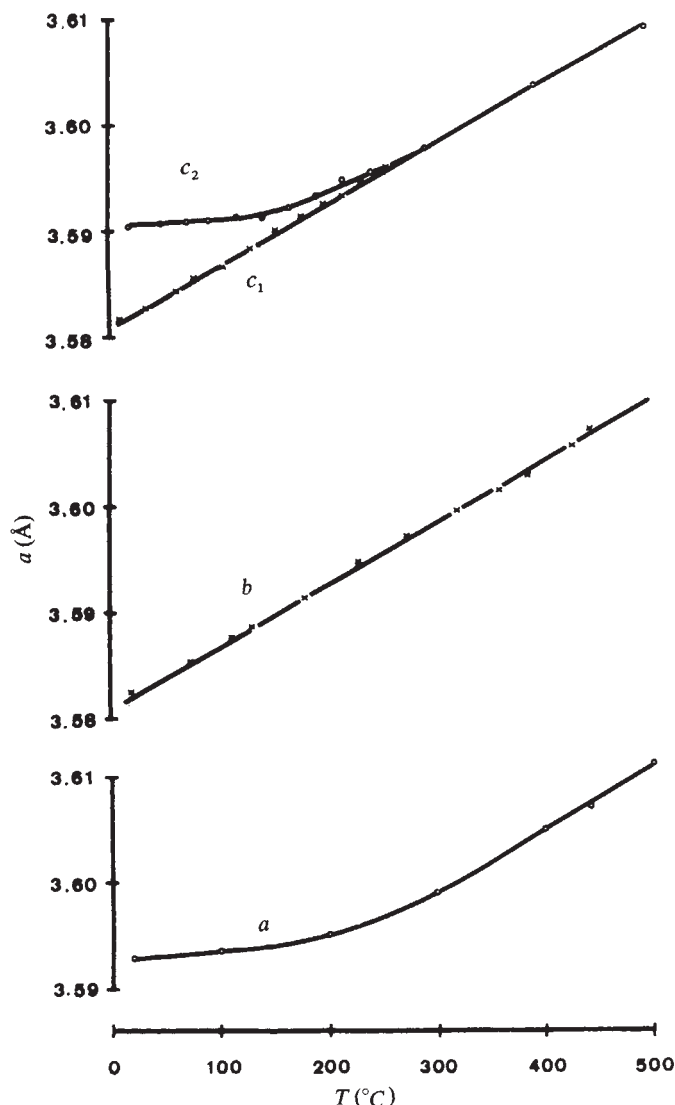


Fig. 3 X-ray lattice parameter variation versus temperature of: a, Fe-Ni Invar alloy before irradiation; b, Fe-Ni Invar alloy after irradiation; c, Santa Catharina meteorite before (c_1) and after (c_2) annealing at 800 °C for 24 h.

ingly low cooling rate which prevails in meteorites when they are inside their parent bodies.

The extensive phase segregation and large proportion of ordered phase observed in the Santa Catharina meteorite are mainly due to its peculiar Ni content (35%), because, as we shall report elsewhere¹⁰, the Invar anomalies which affect both the taenite face centred cubic lattice parameters and the alloy elastic constants also critically affect the phase stability in Fe-Ni alloys.

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Ammonia gas concentrations over the Southern Ocean

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Ammonia is the only common, soluble, basic gas in the atmosphere and as such plays an important neutralising, sometimes rate-determining, role in the chemistry of acid gases such as SO_2 , H_2SO_4 and HNO_3 . It also constitutes a reservoir of labile nitrogen in the atmosphere and so must be included in any attempt to describe the cycling of nitrogen through the global environment. We report here values of ammonia concentration measured in Southern Ocean air which are considerably lower than any previously reported¹⁻⁵. For our analyses ammonia was collected on oxalic acid-impregnated filters and determined colorimetrically. In air apparently devoid of contact with land for several thousands of kilometres of travel the mean concentration of ammonia gas was $0.06 \mu\text{g m}^{-3}$ (STP). Concentrations in air apparently affected by the Australian continent were several times higher than in the maritime air.

Measurements were carried out at the Australian Baseline Atmospheric Monitoring Station at Cape Grim (40.7°S, 144.7°E) on the northwestern tip of Tasmania. This is a coastal site at a latitude where only a few per cent of the Earth's perimeter is land and where shipping traffic is minimal. With no land for several thousands of kilometres in directions between 180° and 290° the station is ideally placed to sample the west-southwesterly winds of the roaring forties, which prevail for more than 60% of the time. There are frequent opportunities for sampling Southern Ocean air free from recent contact with land or human activities.

The method used for measuring the ammonia concentration is a simple but sensitive ring oven analytical technique⁶. Samples are collected over a period of 3 (or more) hours on oxalic acid-impregnated filters using a PTFE (Millipore Fluoropore) prefilter heated ~20 °C above ambient. This system has been calibrated directly down to concentrations of $1 \mu\text{g m}^{-3}$ and found to be quantitative; particulate collection efficiency on the prefilter has been measured and is 100%. Evolution of ammonia from particles collected on the prefilter was tested by periodically inserting an oxalic acid-impregnated filter upstream of the prefilter. No evidence of significant volatilisation was found. Aitken nuclei were measured with an automated nucleus counter referenced to a faithful replica of the counter developed and calibrated by Pollak⁷.

Experiments were carried out between 1 and 14 August 1979. Conditions remained relatively constant throughout, with gently precipitating bands of cumulus cloud passing over the station at intervals of several hours. Temperature remained between 7.4 and 11.9 °C. Wind direction was from the 'baseline sector' (180° to 290°) for 1-4 August and 9-14 August, and from the direction of the Australian mainland (290°-55°) in the intervening period. Surface pressure charts for these three periods are shown in Fig. 1. Records of temperature, wind direction, Aitken nucleus concentration and ammonia gas concentration are displayed in Fig. 2.

The ammonia and Aitken nucleus concentrations seem to be well correlated, with both following closely the observed changes in wind direction. Figure 2 shows that, after allowing for the scatter in the ring oven results, the lowest values occur for winds from the baseline sector. As Aitken nucleus concentration is a sensitive tracer of continental sources in maritime regions, the most reasonable explanation for these variations is that during the period 5-8 August northerly winds brought continental air from the Australian mainland 250 km to the north, whereas the lower levels of ammonia and Aitken nuclei

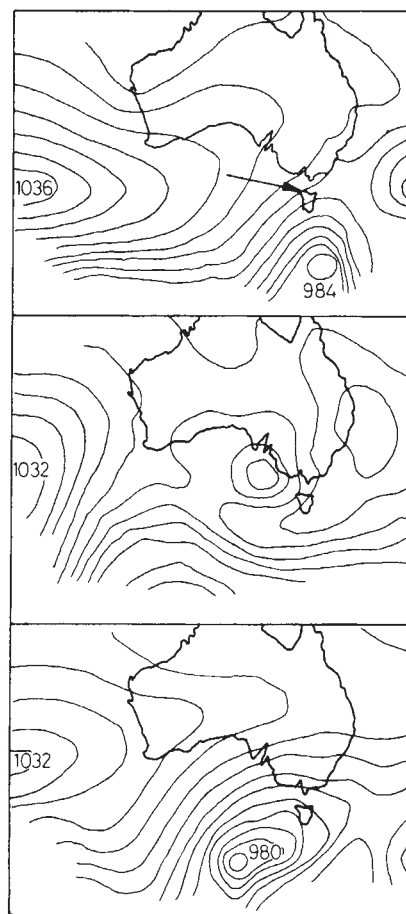


Fig. 1 09.00 EST surface pressure charts. Top, 1 August 1979; middle, 6 August 1979; bottom, 9 August 1979. Isobars are at 4-mbar intervals. The arrow shows Cape Grim.

during 2-4 and 9-14 August correspond with what was a relatively unpolluted southwesterly stream over the Southern Ocean. For 2-4 and 9-14 August we have 41 ammonia gas determinations, giving a mean of $0.06 \mu\text{g m}^{-3}$ (STP) and sample standard deviation of $0.03 \mu\text{g m}^{-3}$ (STP). This is significantly smaller than concentrations reported elsewhere: the range is typically $1-10 \mu\text{g m}^{-3}$ (STP) for the troposphere over Hawaii⁵, central Europe^{1,2}, and the Atlantic⁴ and Pacific Oceans³, with lowest values of $0.2 \mu\text{g m}^{-3}$ (STP)^{3,4}. Our values in the non-baseline condition average $0.18 \mu\text{g m}^{-3}$ (STP), with a sample standard deviation of $0.06 \mu\text{g m}^{-3}$ (STP).

Buch⁸ (in 1920) and others^{4,9} have pointed out that the concentration of ammonia near the ocean surface away from stronger sources (such as continents) should be determined by the composition of the surface waters by a Henry's law relationship. The Southern Ocean area is probably one of the few areas where very long air mass trajectories over the ocean enable this hypothesis to be tested. At present we have no knowledge of the distribution of dissolved ammonia in the ocean upwind of Cape Grim to compare with values predicted from our very low atmospheric concentrations. However, it is of interest to investigate whether the atmospheric values are consistent with the range of surface water ammonia concentrations reported from other locations and with those found in coastal water samples taken at Cape Grim.

From Lau and Charlson⁹

$$p\text{NH}_3 = \frac{K_H K_w [\text{NH}_4^+]}{K_b [\text{H}^+]} \quad (1)$$

where K_H is the Henry's law constant for ammonia, K_w the dissociation constant for water and K_b that for aqueous

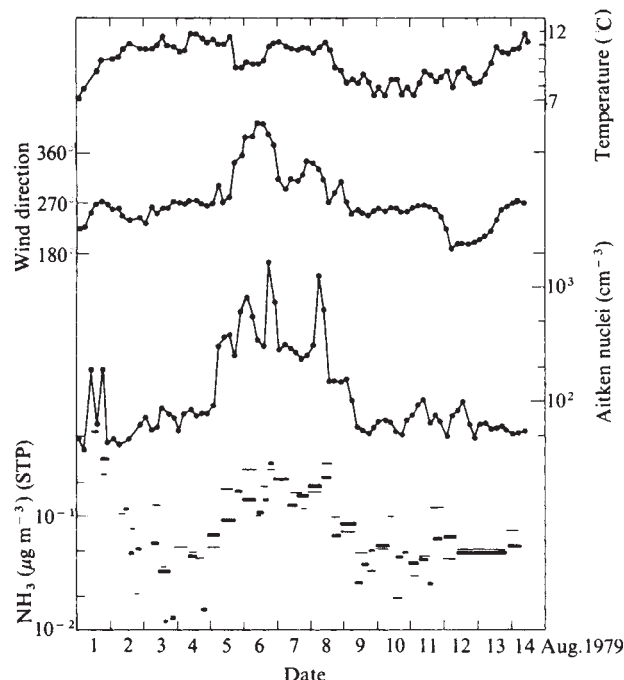


Fig. 2 Records of dry bulb temperature, wind direction, Aitken nucleus concentration and ammonia gas concentration. All points except the ammonia concentrations are 4-hourly averages derived from 24 10-min integrations of the quantity being measured. In the case of ammonia two collectors were used in parallel, with results shown as sets of thick or thin bars. The length of each bar corresponds to the sampling period for that determination.

ammonia. Like Georgii and Gravenhorst⁴, we allow for the fact that the determination of dissolved ammonia does not distinguish between NH_3 and NH_4^+ , by letting

$$a = [\text{NH}_3] + [\text{NH}_4^+] \quad (2)$$

Substitution for $[\text{NH}_3]$ in the expression for K_b gives

$$[\text{NH}_4^+] = \frac{aK_b}{K_b + (K_w/[\text{H}^+])} \quad (3)$$

Putting equation (3) in equation (1) and rearranging gives

$$a = \frac{p\text{NH}_3\{K_w + K_b[\text{H}^+]\}}{K_H K_w} \quad (4)$$

Values of K_H , K_w and K_b and their temperature dependences are available elsewhere^{10,11}. In the present case we use values for 10°C, the mean temperature during the experiments: $K_H = 8.26 \times 10^{-3} \text{ atm mol}^{-1} \text{ l}$; $K_w = 2.92 \times 10^{-15} \text{ mol}^2 \text{ l}^{-2}$; $K_b = 1.57 \times 10^{-5} \text{ mol l}^{-1}$.

For the measured ammonia gas concentration of $0.06 \mu\text{g m}^{-3}$ (STP) and coastal water pH of 8.2, equation (4) implies that, if equilibrium is established, the dissolved ammonia concentration is $0.3 \mu\text{mol l}^{-1}$. This falls within the range of dissolved ammonia values found in the literature, <0.05 to ~ 1 or $2 \mu\text{mol l}^{-1}$, with values from the open ocean lying towards the lower end of this range¹². Our own measurements on three separate samples of coastal water collected during the gas measurements yielded dissolved ammonia concentrations of 0.23, 0.30 and $0.55 \mu\text{mol l}^{-1}$. These results must be qualified, as the analyses were performed two weeks after collection; although the samples were stored untreated in polythene bottles at 0°C and the pH was unchanged over this period it is well-known that storage can affect seawater samples for ammonia analysis¹³.

We therefore conclude that: (1) in winter levels of atmospheric ammonia in Southern Ocean air are 10–100 times less than those reported elsewhere; (2) there are indications that the

mean value observed, $0.06 \mu\text{g m}^{-3}$ (STP), results from a Henry's law equilibrium between atmospheric ammonia and that dissolved in the surface of the ocean.

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Are warm-core eddies unproductive?

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Warm-core eddies¹ are large lenses of subtropical water (radius 100–150 km) wandering through a cooler sea. Figure 1 shows the path of one such eddy (eddy F) in the south-west Tasman Sea. Warm-core eddies in this area are shed by the East Australian Current (EAC)³. Because the EAC is fed mainly by central waters from the western South Pacific^{4,5}, it is low in surface nutrient and generally unproductive^{6,7}. The eddies which it sheds might, therefore, be expected to be equally unproductive. However, the phytoplankton concentration in eddy F increased about fivefold between September⁸ and November 1978⁷. This 'anomaly' is considered here.

Warm-core eddies in the south-west Tasman Sea are recognised by their isothermal layer which extends at the eddy centre

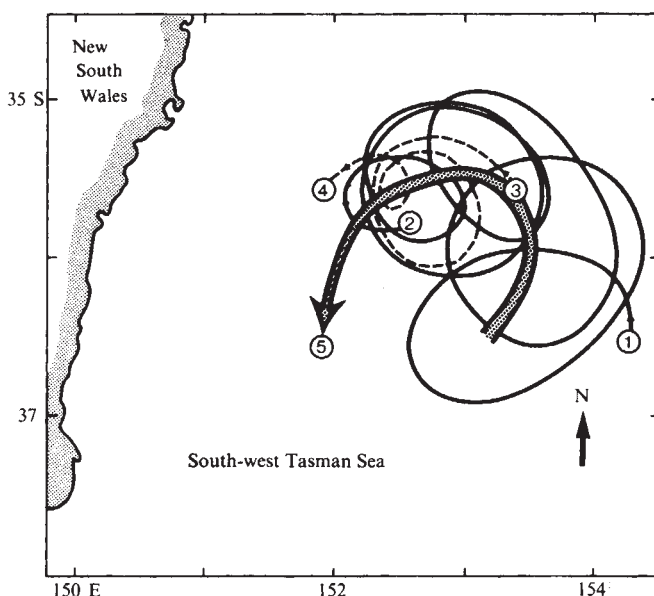


Fig. 1 Probable track of eddy F as deduced from XBT profiles and daily fixes on satellite-tracked buoys drogued at 20 m (ref. 2) (NASA MET SAT NIMBUS-6). Buoy no. 1054 entered the eddy at ① on 16 September and made six complete anticlockwise circuits within the eddy (solid lines) before a systems interruption on 19 October ②. Buoy no. 1132 was released at ③ on 10 October and made two circuits (dashed line) by 19 October ④. The eddy itself seemed to have followed an anticlockwise arc of half a revolution between mid-September and mid-November ⑤.

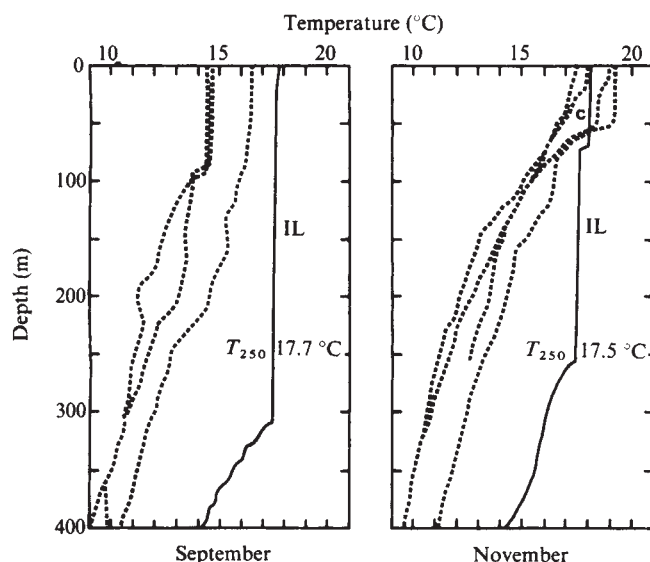


Fig. 2 Temperature profiles taken inside (solid lines) and outside the eddy (dotted lines) in September and November 1978. The eddy is characterised by a deep isothermal layer (IL). In November this was overlain by a slightly warmer 'cap' (c).

to depths as great as 300 m (Fig. 2). A convenient way of identifying particular eddies, and tracking them as they move about, is by their temperature at a depth of 250 m (T_{250}). The centre of eddy F had a T_{250} of 17.5–17.7 °C. The T_{250} at the perimeter of the eddy was ~15 °C. In the following comparison, locations of $T_{250} > 17$ °C are regarded as lying near the centre of the eddy and those at $T_{250} < 15$ °C as lying outside the eddy boundary.

In September 1978 (late winter) the phytoplankton concentration was lower inside the eddy than it was outside (Fig. 3). A second eddy further west (eddy E) had a similar pattern. In November (early summer), the situation was reversed; eddy F now seemed to be far more productive than it had been 2 months earlier (Table 1). The added phytoplankton must either have been brought in from outside the eddy or produced *in situ* from the eddy's own nutrient reserves.

This is undoubtedly the same eddy that was previously under observation in September. Its unique signature is the 17.5–17.7 °C isothermal layer (Fig. 2), and its trajectory had been tracked continuously by 'satellite-buoys' drogued at 20 m: buoys drogued at this depth are relatively unaffected by prevailing winds². The most obvious physical change that had taken

Table 1 Comparison of waters inside and outside eddy F during September and November 1978

Month (1978)	T_{250} (°C)	T_0 (°C)	Parameter DML (m)	NO_3 ($\mu\text{mol N l}^{-1}$)	F_M (TU)
September					
Inside	>17	17.7	215–320	2.9–3.2	100–200
Outside	<15	15.3–20.2	37–88	1.1–3.3	200
November					
Inside	>17	18.0–18.2	60–65	0.3–0.8	200–600
Outside	<15	17.5–18.2	60	0.6	200–400

Depth of the mixed layer (DML), T_{250} and T_0 were taken from XBT records. Nitrate was determined by the strychnidine method⁹ using Nansen bottle samples; the values shown are averages for the mixed layer. F_M (*in vivo* chlorophyll *a* fluorescence maximised with 'Diuron'^{10,11}) was measured in a flow-through system with injected dose to give 6 μM concentrations. The source water was pumped through the ship's hull from ~2 m depth. F_M is given in standardised *in vivo* chlorophyll *a* fluorescence units¹² (TU) and is taken as an index of phytoplankton biomass.

place in the interim was the development of a warmer 'cap' (18.0–18.2 °C) extending to a depth of 60–65 m (Table 1). The depth of mixing had changed from >300 m (the depth of the deep isothermal layer) to <100 m (the depth of the surface cap). Did this cap develop *in situ* by surface warming, or was it derived from 'outside' surface water which had washed across the top of the eddy?

We first consider the possibility of advection. The EAC tends to advance southwards in the summer, sometimes 'recapturing' eddies shed the previous winter, particularly those located north of Sydney (C. J. Nilsson and G.R.C., unpublished data). However, in November 1978, the EAC was located 640 km north of eddy F. Had warm water from another source washed across the top of eddy F we would have expected the buoys adrift within the eddy (Fig. 1) to be ejected: they were not. Phytoplankton concentrations immediately north of eddy F were much too low to represent a likely source of advected water (Fig. 3). Only beyond its southern limits were November phytoplankton concentrations of the same order as those within the eddy, and here the water was too cold to be a likely source.

For these reasons, we have concluded that the development of the summer cap in eddy F, and the associated increase in phytoplankton concentration, were processes that took place *in situ*, within the confines of the eddy. How did they take place?

Data presented in Table 1 provide a contrast between the conditions prevailing in September and November, both for eddy F and the surrounding waters. The focus is on winter mixing and thermocline formation. In the eddy, winter mixing

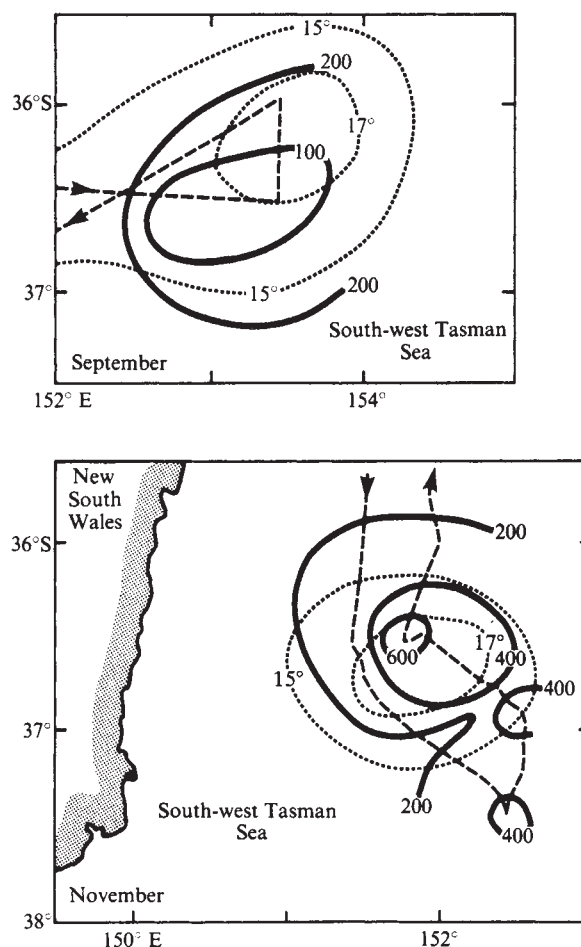


Fig. 3 'Surface' phytoplankton concentration in the region of eddy F in September and November 1978. The perimeter of the eddy is indicated by the 15 °C isotherm (at 250 m), the centre by the 17 °C isotherm. The phytoplankton contours are *in vivo* chlorophyll *a* fluorescence (F_M) measured underway along the cruise track (for details see Table 1).

had extended deeper, distributing inorganic nitrate fairly uniformly throughout the isothermal layer (Fig. 4). In September, deep-lying stores of NO_3 were being mixed into the euphotic zone of the eddy. By November, most of that which had been introduced in this way was now present in the form of phytoplankton biomass. The mean NO_3 concentration in the upper mixed layer of the eddy (60–65 m) had decreased from 2.9–3.2 $\mu\text{mol N l}^{-1}$ in September, to 0.3–0.8 $\mu\text{mol N l}^{-1}$ in November. The September and November status of eddy F are described in detail elsewhere^{7,8}, as are the conditions in October and December^{13,14}.

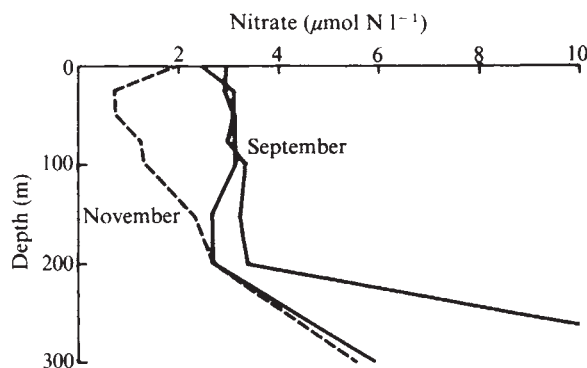


Fig. 4 Nitrate (NO_3) profiles for stations inside the eddy in September and November. September profiles in the eddy displayed nitrate concentrations of the order of 3 $\mu\text{mol N l}^{-1}$ to 200 m. The November profile indicated that more than half of this had been consumed.

These phenomena are explained by Sverdrup's model¹⁵ of environmental control of the spring bloom¹⁶. The Sverdrup model explains the onset of a bloom in relation to the depth of the mixed layer and to the 'critical depth', that is, where respiration losses for the water column equal gains by photosynthesis. In the south-west Tasman Sea, the 1% light level is at a depth of about 75 m (ref. 17), much shallower than the depth to which eddy F mixed in September (250–350 m). Consequently, the standing crop could not increase. Nutrients were distributed throughout the mixed layer, but remained unused for plant production during this light-limited mixing era. When in contact with a mass of air cooler than that at its origin, the eddy took longer to come to thermal equilibrium than did the surrounding sea. Its surface water continued to cool and sink to the level of the permanent thermocline. This process of convective overturn¹ caused deep mixing which persisted through the spring, by which time surface waters outside the eddy had begun to warm. By November, the eddy mixing cycle had ended and it had developed a summer cap (Fig. 2).

We conclude, therefore, that the production of a warm-core eddy is greater than that of the water mass from which it is derived because prolonged surface cooling and deep convective mixing convey previously unavailable nutrients to the surface. This results from the movement of the eddy into a location where the atmosphere is significantly colder.

When eddies are isolated from the surrounding sea, their production is limited by the concentration of nutrients in the winter isothermal layer. Each subsequent year this would be reduced by annual phytoplankton blooms, much of which material would presumably be lost to the system by detrital fallout. In addition to this organic loss, the eddy would lose heat and eventually come to equilibrium with atmosphere and surrounding sea. We would expect these two factors to combine to reduce progressively the scale of consecutive phytoplankton blooms for as long as the eddy remained a discrete water body.

Our studies^{7,8,13,14} show that warm-core eddies can be useful systems in which to study pelagic processes. They can readily be

identified, marked and tracked through time, and are sometimes so discrete that a pulse of primary production can be followed through the food chain.

This letter has dealt principally with production in the eddy proper. Unpublished information indicates that plankton are concentrated at the frontal boundary of the eddy. The factors controlling these concentrations are being studied.

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A revised age for the Donegal granites

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The Donegal granites in north-west Ireland are among the best mapped granitic intrusions in the world¹. They form part of a broad suite of granites intruded during the Palaeozoic but within a relatively small area encompass a wide range of emplacement features. A published Rb–Sr composite whole rock isochron for these granites defined a date of 487 ± 5 Myr (refs 2, 3) whereas K–Ar ages exhibited a spread from 365 ± 8 to 412 ± 8 Myr (refs 1, 4). (All age data are normalized to recently recommended decay constants⁵). Although some doubt was cast on the older Rb–Sr date by Pitcher and Berger (ref. 1, p. 90) and Leake⁶ it has generally been accepted as a more valid estimate of the emplacement age^{2,3,7–9}. Here we summarize the results of Rb–Sr and U–Pb isotopic analyses recently carried out which indicate that the Rosses, Ardara, Trawenagh Bay and Main Donegal intrusions were, in fact, all emplaced close to 400 Myr ago.

Rb–Sr isotopic analyses have been made on more than 70 whole rocks (5–10 kg size) and mineral separates and U–Pb isotopic analyses on zircons and a monazite using methods outlined elsewhere¹⁰. The complete results together with their petrogenetic implications will be presented elsewhere. Here we present a summary of the regression results¹¹ obtained on Rb–Sr age data for those portions of each of four of the main intrusions which best define perfect isochrons. The sum of the squares of the residuals (SUMS) indicates the goodness of fit of the data relative to that expected from the analytical uncertainties. For the Ardara, Rosses and Trawenagh Bay intrusions the best fitting data come from the central portions of the intrusions and the aplites which exhibit a wider spread of Rb/Sr ratio. The outer portions of these intrusions (for which results are not

shown) apparently have variable initial $^{87}\text{Sr}/^{86}\text{Sr}$ ratios (compare with refs 12–14). Samples from the Main Donegal Granite had a restricted range of Rb/Sr and the results obtained from mineral separates and the corresponding whole rock samples are given.

The Rosses Complex whole rock data yield a well defined isochron age of 404 ± 3 Myr which is effectively identical to the age of 405 ± 3 Myr obtained for Trawenagh Bay intrusion (Fig. 1, Table 1). The Ardara data scatter significantly at the 5% level and the best fitting age of 405 Myr should be regarded as an approximation to the age of intrusion. Use of York's¹¹ "scatter error" would give a 2σ uncertainty of ± 15 Myr. The five mineral separates (biotite, muscovite, K-feldspar, plagioclase and apatite) and whole rock data points for sample D/12/4 of the Main Granite define an isochron date of 388 ± 3 Myr. This probably represents the time when the intrusion cooled. A second sample (D/17/1) gives similar results for muscovite–biotite–apatite–whole rock, but K-feldspar and plagioclase scatter slightly away from the other points.

A U–Pb isotopic analysis of monazite from the Trawenagh Bay pluton yielded concordant $^{238}\text{U}/^{206}\text{Pb}$, $^{235}\text{U}/^{207}\text{Pb}$ and $^{207}\text{Pb}/^{206}\text{Pb}$ ages whose mean is 411 ± 2 Myr (2σ). U–Pb data for zircons, however, are all discordant with marked inherited radiogenic Pb components analogous to many Caledonian granites from Scotland, north of the Highland Boundary Fault¹⁵. These data strongly suggest melting or assimilation of Precambrian crustal materials or their sedimentary or magmatic derivatives in the genesis of the granites. In this respect it is of interest that the initial $^{87}\text{Sr}/^{86}\text{Sr}$ ratios of the magmas were low (Table 1) which further restricts the sources to having low Rb/Sr ratios. Hence the Donegal granites were generated from low Rb/Sr, low $^{87}\text{Sr}/^{86}\text{Sr}$ crust, possibly in combination with mantle or basaltic lower crustal materials (compare with ref. 10 and ref. 1, Ch. 16).

Pitcher and Berger (ref. 1, p. 90) outlined the probable chronology of these Donegal granites in the order Ardara, Rosses, Main Granite and Trawenagh Bay. The best age data here have been obtained on the Rosses and Trawenagh Bay intrusions which yield similar Rb–Sr whole rock dates indicating that it is exceedingly unlikely that the former preceded the latter by more than 5 Myr. The slight difference between the U–Pb monazite date and the Rb–Sr whole rock date for Trawenagh Bay could merely reflect the uncertainty of absolute calibration of each dating system. The minerals–whole rock Rb–Sr age of 388 ± 3 Myr obtained from the Main Donegal Granite places a minimum on the emplacement age of this intrusion. The highly foliated character of this granite makes it clear that mineral dates should be interpreted with caution. The results from

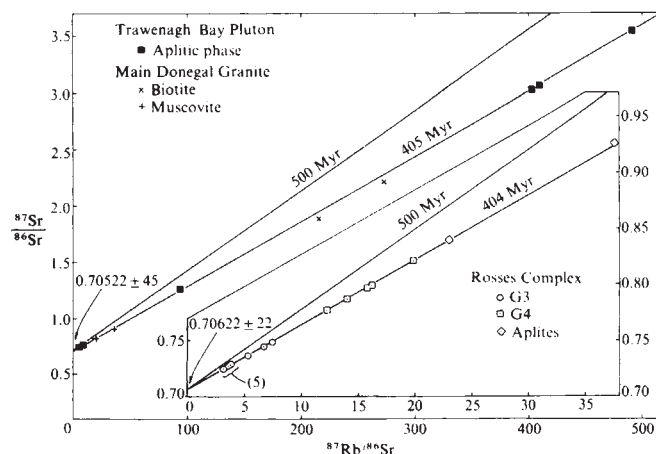


Fig. 1 Rb–Sr isochron diagram for Trawenagh Bay (aplitic phase) and Rosses (G3, G4 and aplites) whole rock samples, and biotites and muscovites from the Main Donegal granite. Reference isochrons are shown.

Ardara indicate that this intrusion was emplaced at about the same time as the others.

The results presented here are inconsistent with previous Rb–Sr age studies^{2,3} in which the Rb–Sr data were interpreted as indicating an age of 487 ± 5 Myr for the Main Donegal, Trawenagh Bay, Thorri and Rosses granites, and also inconsistent with a time lapse of ~40 Myr between intrusion of the Rosses granite and its aplites². Our results are not inconsistent with those of Long¹⁶ but do resolve the ages more satisfactorily and indicate that the whole rock Rb–Sr isochron date of 352 ± 16 Myr (decay constant not given) he obtained for the Main Donegal Granite does not reflect the emplacement age.

The results presented here indicate that at least a major portion of the magmatism in Donegal took place close to 400 Myr ago and that these granites are temporally related to the 'newer' and 'last' Granites as defined by Read¹⁷. Several implications follow from this. The major deformation and metamorphism in Donegal could be younger than previously thought³, and there is no need to invoke diachronous deformation between County Mayo and County Donegal on the basis of the radiometric age of the Donegal granites^{7,9}. Similarly the need to fit voluminous but localized intrusions of granite at ~490 Myr into plate tectonic models of the Caledonides^{7,8} can be done away with. Like most Caledonian granite magmatism the Donegal granites seem to relate temporally to late- or post-closure processes.

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Table 1 Results of regression analysis on Rb–Sr data for Donegal granites

Samples	n*	Age $\pm 2\sigma$ (Myr)†‡	($^{87}\text{Sr}/^{86}\text{Sr}$) _i $\pm 2\sigma$	SUMS
Rosses G3, G4, ap. w.r.	15	404 ± 3	0.7062 ± 2	20.4
Trawenagh Bay ap. phase w.r.	6	405 ± 3	0.7052 ± 4	4.60
Main Granite D/12/4 w.r. & m.s.	6	388 ± 3	0.70654 ± 4	6.76
Main Granite D/17/1 w.r. & m.s.	6	386 ± 4	0.70604 ± 1	271§
Ardara G2b, ap. w.r.	7	405 ± 5	0.70655 ± 6	48.0§

ap, aplitic; w.r., whole rock(s); m.s., mineral separates, uncertainty in $^{87}\text{Rb}/^{86}\text{Sr} = 0.7\%$ (1σ) and in $^{87}\text{Sr}/^{86}\text{Sr}$ averages 0.01% (1σ), NBS987 gives $^{87}\text{Rb}/^{86}\text{Sr} = 24.20 \pm 0.05$ and $^{87}\text{Sr}/^{86}\text{Sr} = 1.2004 \pm 0.0005$.

* Number of points.

† $\lambda^{87}\text{Rb} = 1.42 \times 10^{-11} \text{ yr}^{-1}$.

‡ Errors refer to least significant digits and are of York's¹¹ a priori type.

§ Significantly large at 5% level.

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The connectance of real ecosystems

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In the work of Gardner and Ashby¹, and May² it has been shown that ecosystem stability (in the sense of Liapunov stability of equilibria³) imposes nontrivial constraints (summarised below) on ecosystem structure, but little has been done to relate this work to observations. One such study was undertaken by Rejmánek and Starý⁴, who found in a collection of plant-aphid-parasitoid food webs that connectance C (roughly speaking, the fraction of pairs of species that directly interact) decreases like S^{-1} as species richness S (the total number of species) increases. Here I analyse a sample of community food webs (the webs of ref. 4 are sink webs⁵), and find a somewhat slower decrease of connectance with increasing species richness. This behaviour suggests, within the context of May's theory², that complex ecosystems will tend either to be more fragile or to have weaker interspecific interactions than simpler ones.

Connectance, as it occurs in May's theory, is defined precisely as the fraction of non-zero off-diagonal elements of the community matrix². One can also define⁶ an 'average strength', i , of interspecies interactions. Then, for a certain ensemble of randomly constructed systems, May found that the community matrix will almost certainly be stable if

$$i < (SC)^{-1/2} \quad (1)$$

and it will almost certainly be unstable if

$$i > (SC)^{-1/2} \quad (2)$$

with the transition from stability to instability being very sharp if $S \gg 1$.

A food web, which specifies the feeding relationships in a community, can be used to estimate the connectance of the community matrix as follows. First, if species a feeds on species b then the elements A_{ab} and A_{ba} of the community matrix A will certainly be non-zero. If we know all the feeding relationships in a community, we can determine in this way a set of community matrix elements which are certainly non-zero, whence a lower bound C_1 on the connectance. Second, some species will directly interfere with one another, generally in competition for shared

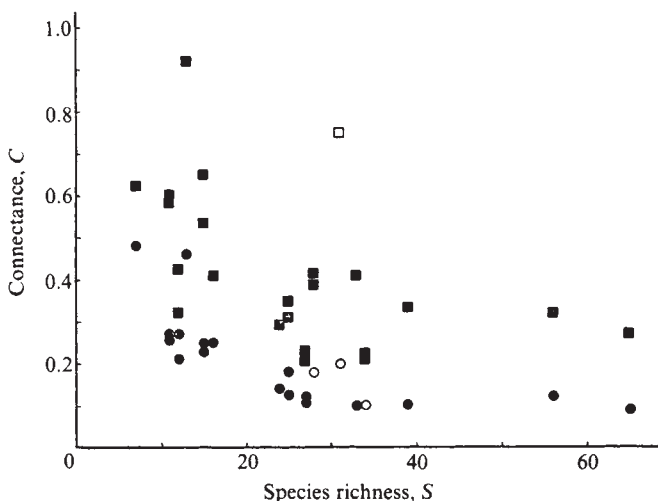


Fig. 1 The estimates C_1 (circles) and C_u (squares), plotted against species richness S for the 24 community food web versions compiled by Cohen⁵. Each open symbol corresponds to two food web versions.

resources. Thus if species a feeds on species b and species c also feeds on species b , then species a and c may engage in interference competition with one another (although they may instead manage to avoid such competition by, for instance, partitioning the food resource in some way). If these two species do interfere with one another, then the elements A_{ac} and A_{ca} will be non-zero. Assuming that any two species which share any food resource interfere with one another, we can determine in this way an additional set of potentially non-zero community matrix elements which, added to the feeding matrix elements previously obtained, yield another estimate C_u of the connectance.

In general there will be additional interactions in a community, due to, for instance, competition for resources other than food, parasitism, and mutualism. If we assume that these interactions are less common than interactions involving food resources, then C_u can be regarded as something like an upper bound on connectance. Otherwise, it can simply be regarded as an estimate which is arrived at in a well-defined way.

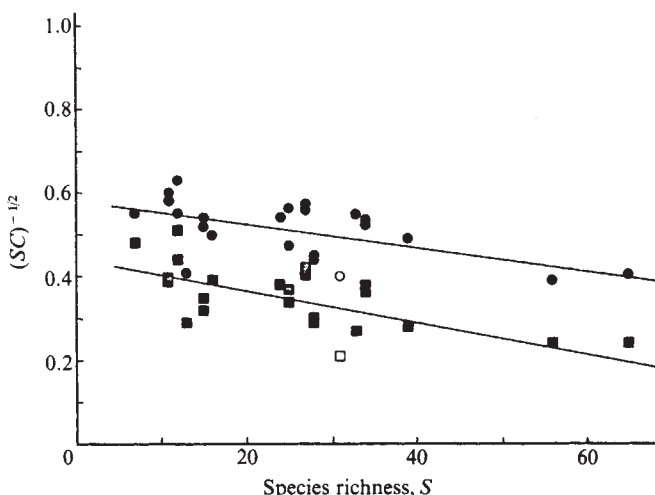


Fig. 2 The estimates $(SC_1)^{-1/2}$ (circles) and $(SC_u)^{-1/2}$ (squares), plotted against species richness S for the 24 community food web versions compiled by Cohen⁵. Each open symbol corresponds to two food web versions. The straight lines are linear regressions of $(SC_1)^{-1/2}$ and $(SC_u)^{-1/2}$ on S .

Cohen⁵ has extracted 24 community food web versions from literature data. While admittedly far from perfect (especially with regard to grouping species into categories), this collection is probably the best statistical base presently available for food web studies. In several cases Cohen gives two (or even three) food web versions for one community; thus one can distinguish for some communities between food web versions of type A, which include only feeding relationships of which the field investigator was certain, and food web versions of type B, which include additional feeding relationships considered probable by the observer. It is not entirely clear whether the most sensible statistical sample is the full collection of all 24 food web versions, the set of 14 type A food web versions, or the set of 14 type B food web versions. Fortunately, the results of this investigation are essentially the same for all three samples.

The connectance estimates C_1 and C_u for Cohen's 24 community food web versions are plotted against species richness S in Fig. 1. There is a clearly discernible tendency for both estimates to decrease as S increases. However, for decreasing connectance alone to account for the stability of complex systems, C must decrease at least as fast as S^{-1} , which is not the case here.

This is made explicit in Fig. 2, which is a plot of the two estimates for $(SC)^{-1/2}$ (hence, according to equation (1), the upper bound on the interaction strength i which allows stability) against S for the 24 Cohen community food web versions. The straight lines are linear regressions on S of the two estimates;

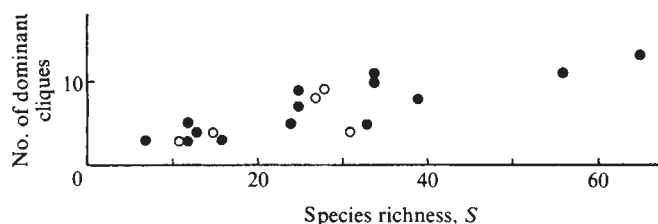


Fig. 3 The number of dominant cliques plotted against species richness S for the 24 community food web versions compiled by Cohen⁵.

both slopes are negative and significantly different from zero (Student's t -test, $P=0.0036$ for $(SC_1)^{-1/2}$, $P=0.0006$ for $(SC_2)^{-1/2}$). The significance of this, at least within May's framework², is that unless average interaction strength tends to decrease with increasing S , systems with larger S will be closer to the critical value $i(SC)^{1/2}=1$ which marks the transition to instability; one expects that such systems would be more fragile in the sense of susceptibility to external perturbations.

Most ecologists would probably expect a decrease in C as S increases, on the basis that ecosystems tend to be organised into

relatively small 'guilds' of species, with most interactions taking place within guilds^{2,6-9}. This idea can be made precise and tested as follows. Define a 'clique' as a set of species with the property that every pair of species in the set has some food resource in common, and define a dominant clique as a clique which is contained in no other clique^{5,10}. These dominant cliques may appropriately be regarded as 'guilds'. In Fig. 3 the number of dominant cliques is plotted against species richness S for the 24 Cohen community web versions. The number of dominant cliques increases with S , although perhaps not as rapidly as one might have expected.

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Effects of leaf age and plant life history patterns on herbivory

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Current theories on plant-herbivore interactions suggest that plant species of different successional status and leaves of various ages differ in their degree of ephemerality and predictability to herbivores, and will therefore exhibit different anti-herbivore characteristics¹⁻⁶. Old leaves and leaves of mature forest plants are expected to be better defended than ephemeral young leaves and leaves of early successional plants. These predicted patterns of plant defence and the resultant patterns of insect grazing are not well documented for natural communities. Field studies have shown that mammalian herbivores in a tropical forest prefer young leaves⁷ and that insect grazing in a temperate forest is heaviest on the young leaves⁸. Laboratory studies have shown that late successional species⁹⁻¹² or plants with certain chemical defences¹³⁻¹⁷ are less palatable for generalist herbivores. Laboratory results depend, however, on the particular herbivore tested, and may not accurately predict rates of herbivory in natural systems. Here I report on rates of herbivory on young and mature leaves from tree species with different life history patterns. Grazing rates (% leaf area eaten per day) on mature leaves of fast growing, shade-intolerant species (pioneers) were an order of magnitude greater than those on slow growing, shade-tolerant species (persistents). Young leaves in both groups of species suffered significantly greater grazing damage than mature leaves.

For this study, tree species were classified into two groups, pioneers or persistents. Persistent species tolerate shade as seedlings and saplings, whereas pioneer species are unable to survive in the shade¹⁸⁻²³. Pioneer species may differ from persistent ones in antiherbivore characteristics because of these differences in their life history patterns. Persistent species grow slowly for long periods of time in the understory before a light gap opens above them, and thus they may be a more predictable resource for herbivores and may be expected to possess costly and effective defences. Mature leaves may be tough and contain

substances that reduce digestibility, such as tannins and resins^{1-4,24,25}. In contrast, saplings of pioneer species may invest in metabolically less expensive toxic chemicals and instead rely primarily on escaping herbivory through fast growth and a patchy occurrence in light gaps¹⁻⁴. Young expanding leaves of both types of plants are a nutritious^{5,26,27} and ephemeral resource, and presumably are not heavily defended by chemicals, especially not substances that reduce digestibility (refs 1-6 but see 7, 28). Synchronous flushing of young leaves by individuals or populations could lead to temporal escape as well as satiation of herbivores^{5,29,30}.

Rates of insect grazing were evaluated for 235 saplings (1-2 m high) of 11 pioneer and 16 persistent species growing in 13 light gaps in the semideciduous forest of Barro Colorado Island, Panama³¹. These 27 species of canopy trees were chosen because both adults and saplings were abundant. I measured total leaf area and area of holes made by herbivores of 1,745 marked young and old leaves at 2-week intervals for three months at the beginning of the rainy season (April-July 1977). Young leaves were measured from the time they emerged from the bud until they were fully expanded and had mature characteristics. Grazing rates were expressed as the percentage of the entire leaf area eaten per day which controls for leaf area in expanding young leaves (see Table 1 for a description of rate measures).

The data on grazing rates (Table 1) suggest that there is a strong relationship of both life history patterns and leaf age with grazing by herbivores. For 23 of the 27 species, there is no overlap between the distributions of pioneer and persistent species in the mean grazing values on mature leaves. There is an order of magnitude difference between the mean grazing rate on mature persistent leaves and that on either mature leaves of pioneers or young leaves of persistents. There is a smaller but significant difference ($P<0.01$) between the grazing rates on young and mature pioneer leaves. There is no significant difference between the grazing rates on young leaves of pioneer and persistent species.

These results support predictions of current hypotheses¹⁻⁴ which propose that investment in antiherbivore defences will reflect the degree of predictability of each plant or plant part as a resource for herbivores. Low rates of grazing on mature persistent leaves suggest that they are well defended from herbivores, possibly by digestibility reducing substances³. High rates of herbivory on young leaves and pioneer plants suggest that if escape in time or space is operating, it is apparently not so

Table 1 Grazing rates (% leaf area eaten per day) on young and mature leaves of pioneer and persistent species

Persistent species*	Mature leaves	Young leaves
<i>Fareamea occidentalis</i>	0.002	0.069
<i>Virola sebifera</i>	0.002	0.108
<i>Prioria copaifera</i>	0.002	0.014
<i>Swartzia simplex</i>	0.003	2.504
<i>Simarouba amara</i>	0.003	0.026
<i>Trichilia cipo</i>	0.003	0.522
<i>Poulsenia armata</i>	0.004	0.027
<i>Desmopsis panamensis</i>	0.004	0.783
<i>Tachigalia versicolor</i>	0.005	0.775
<i>Tetragastris panamensis</i>	0.005	1.477
<i>Protium tenuifolium</i>	0.008	0.928
<i>Hirtella triandra</i>	0.042	0.120
<i>Quararibea asterolepis</i>	0.114	0.316
<i>Alseis blackiana</i>	0.136	0.096
<i>Zanthoxylum panamense</i>	0.136	0.701
<i>Cupania sylvatica</i>	0.311	1.151
Mean†	0.041 ^{abc}	0.631 ^c
Standard error‡	0.024	0.151
No. of species	16	16
No. of plants	120	115
No. of leaves	452	509
Pioneer species	Mature leaves	Young leaves
<i>Didymopanax morototoni</i>	0.007	0.001
<i>Croton bilbergianus</i>	0.025	0.310
<i>Zanthoxylum belizense</i>	0.081	0.624
<i>Spondias radlkoferi</i>	0.186	1.492
<i>Miconia argentea</i>	0.189	0.509
<i>Ochroma pyramidale</i>	0.191	0.178
<i>Alchornea costaricensis</i>	0.210	0.818
<i>Luehea seemannii</i>	0.456	1.108
<i>Cecropia insignis</i>	0.797	0.111
<i>Trema micrantha</i>	1.071	0.053
<i>Cecropia obtusifolia</i>	2.267	1.299
Mean†	0.466 ^{ab}	0.663 ^a
Standard error‡	0.162	0.161
No. of species	11	11
No. of plants	102	105
No. of leaves	386	398

Grazing rates for each leaf were computed as $100 \times (\text{change in hole area}) / ((\text{total leaf area}) \times (\text{no. of days between observations}))$. Significant distortion in the percentage of leaf area eaten does not occur during leaf expansion. Holes of known diameter were punched in 209 young leaves of five pioneer species (*Cecropia insignis*, *Croton bilbergianus*, *Miconia argentea*, *Trema micrantha* and *Zanthoxylum belizense*) and five persistent species (*Desmopsis panamensis*, *Protium tenuifolium*, *Trichilia cipo*, *Virola sebifera* and *Zanthoxylum panamense*). A regression of the percentage change in hole area against the percentage change in total leaf area over time gave a slope not significantly different from 1 ($P < 0.05$, $r^2 = 0.88$ around $\beta = 1$).

*Species are ordered by grazing rates on mature leaves.

†Values followed by the same letter are significantly different, $P < 0.01$ for a and $P < 0.001$ for b and c . Significance levels were determined by a nested 2-way ANOVA considering leaves as replicates on a transformation of the data: $\ln(1,000 \times \text{rate} + 1)$. Although the data were not normally distributed (as a result of many zero values), ANOVA seemed the most appropriate statistical method. The results were not changed when leaves with values of zero damage were excluded. Similar results were obtained when a new rate was calculated by averaging the actual leaf and hole areas per plant rather than the percentage eaten. This second rate of herbivory was computed as: $((\sum_i \text{change in hole area}) \times n_i) / ((\sum_i \text{leaf area}) \times (\sum_i \text{no. of observation days}))$, where i is the i th plant and n the number of leaves. This measure of grazing is less sensitive to the contribution by young leaves that were completely eaten.

‡Standard error of the species means.

effective in reducing losses to herbivores. The magnitude of the differences in grazing rates that I observed emphasises the range of susceptibility to herbivores that exists in natural communities and the presumed variety and effectiveness of different anti-herbivore characteristics.

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Female mimicry in male bluegill sunfish—a genetic polymorphism?

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When male reproductive success depends on male-male competition and aggression, individuals which are at a competitive disadvantage sometimes adopt an entirely different constellation of reproductive behaviours. When such alternative reproductive patterns are practised opportunistically, as when a defeated territorial male adopts a parasitic role, or as part of a developmental sequence, as when younger males are satellites, they can be considered to be part of a single lifetime reproductive strategy. In contrast, when alternatives are available but individuals practise only a single reproductive option throughout their lifetime, the possibility of a genetic polymorphism can be considered. Such genetically mediated alternative reproductive strategies have been hypothesised¹, but the ontogeny of supposed alternative reproductive strategies is seldom known². This letter describes two reproductive patterns in bluegill sunfish (*Lepomis macrochirus*), a nesting male strategy and a female mimic strategy, and demonstrates that an individual male does not practise both reproductive strategies.

Alternative reproductive patterns have been described as 'sneak', 'satellite', or 'parasitic' behaviour, and have been found in a wide variety of taxonomic groups. These alternative patterns are often 'submissive', and can include the display of female characteristics which serve to reduce male aggression³⁻⁵. The most detailed cases of female mimicry as a reproductive option have been reported in scorpionflies⁶, where males increase their frequency of mating by obtaining nuptial gifts from other males; salamanders⁷, where males stimulate rivals to waste their gametes; and fishes, where female mimics attempt to gain access to females attracted to territorial males. In these cases of detailed female mimicry, individuals either were not identified or were observed to practise both typical male behaviour and female mimicry.

For fishes, female mimicry was first described in the 10-spined stickleback, *Pungitius pungitius* L.⁸, and the leaf fish *Polycentrus schomburgkii*⁹, in aquaria. Female mimics have recently been observed in the field in a Michigan population of *Lepomis macrochirus*¹⁰, and in benthic Mediterranean fishes of the genus *Tripterygion*¹¹. The behavioural observations reported here were made while snorkelling or scuba diving in Lake Cazenovia, New York, during the summers of 1976, 1978 and 1979, as part of a long-term study of the mating system of *Lepomis macrochirus*. Following all behavioural observations, individuals were captured with a hand net and stripped of their gonadal products to verify their sex. Because of the difficulty of identifying female mimics in the field, female mimicry in fishes may be much more common than the few reported instances indicate.

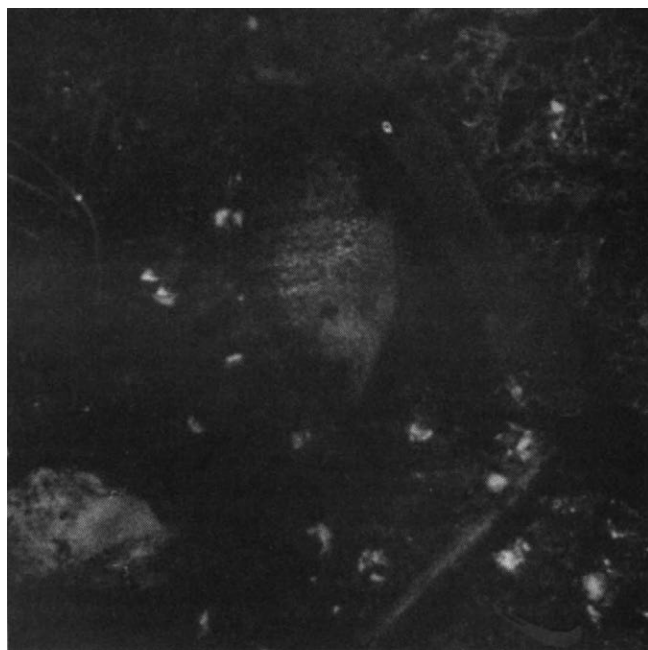


Fig. 1 Nesting male (left), female (right, depositing eggs), and female mimic (small dark fish, between) simultaneously spawning. The line drawing shows the position of the fish—the female mimic is almost lost in the shadow from the larger nesting male.

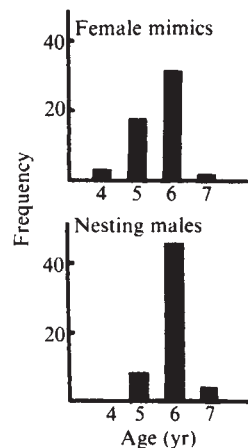


Fig. 2 Age distributions for female mimics (top) and nesting males (bottom).

As is typical of the species, male *Lepomis macrochirus* in Lake Cazenovia occupy nesting territories in densely packed colonies which may contain several hundred nests. Males compete aggressively for position within these colonies, and the ability of a male to maintain residency on a nest is related to its size (unpublished data). This is consistent with aquarium studies of *Lepomis macrochirus* and other sunfishes where body size has been found to be an important determinant of success in agonistic encounters¹²⁻¹⁵. Males which mimicked female behaviour in 1978 ($\bar{x}$ = 126 mm, s.d. = 9 mm, n = 20) were much smaller than nesting males ($\bar{x}$ = 190 mm, s.d. = 13 mm, n = 45), and were never seen to compete aggressively with them. Although the size distribution of female mimics was disjunct from that of nesting males, it did overlap with the lower end of the distribution for spawning females ($\bar{x}$ = 156 mm, s.d. = 19 mm, n = 45).

An actively spawning *Lepomis macrochirus* colony is easily recognised because hundreds of females and males without nests typically aggregate in the water column. To initiate courtship, a female leaves this aggregation and swims towards a nesting male while displaying a coloration pattern (dark background, dark eyes, and pronounced vertical barring) known to reduce aggression in the nesting males of another sunfish, *Lepomis gibbosus*¹⁶. Once the female is in the nest, the male begins to swim in tight circles, turning, with the female following. Every few seconds as the pair turns, the female lies on her side, presses her genital pore against that of the male, quivers, and releases eggs that the male simultaneously fertilises¹⁷.

The behaviour of female mimics is essentially the same as that of functional females. Female mimics do not attempt to construct or defend nest sites, are found in the predominantly female aggregations, and solicit nesting males for courtship. These small males approach the aggressive, territorial males without hesitating or retreating, adopt the dark, barred coloration, and turn in the nest with the resident males. Surprisingly, in some colonies the majority of 'spawning' pairs were nesting male-female mimic pairs. Although this type of 'homosexual' behaviour would not seem to be immediately adaptive, the adaptive significance of female mimicry becomes evident when female mimics spawn as part of a trio (Fig. 1) or larger group which includes a functional female. During these spawning bouts, any eggs fertilised by female mimics are abandoned to the care of the nesting males.

As in the primary males of the bluehead wrasse *Thalassoma bifasciatum*, where sperm competition is also likely to be important¹⁸, the testes of female mimic *Lepomis macrochirus* occupy a greater proportion of their body weight (4.2%) than do the testes of territorial males (1.9%). These large, sperm-laden testes contrast strongly with immature testes or the atrophied testes found in non-breeding individuals. Although released sperm were not visible for either female mimics or nesting males during spawning bouts, a slight pressure to the abdomen of a

female mimic would invariably release copious quantities (compared with a nesting male) of sperm. In the laboratory, these sperm were found to fertilise eggs as readily as those of nesting males. Given that large amounts of active, fertile sperm are present and easily released mechanically, it is likely that during spawning bouts female mimics were releasing sperm and were fertilising eggs. An extremely complex and unusual evolutionary scenario would be required to explain why individuals with exceptionally developed and spermatogenically active testes would adopt an elaborate reproductive pattern, gain access to egg-releasing females, and then fail to release sperm.

Although female mimics are much too small to successfully practise nesting male behaviour during a given breeding season, these small males might continue to grow and eventually reach nesting male size. In such a case, where female mimics represented a developmental stage of nesting males, female mimics should show a younger age distribution. Fortunately, fishes, especially temperate, freshwater fishes offer a distinct advantage over many other vertebrates for studies of the ontogeny of alternative reproductive strategies because they can be accurately aged using the growth characteristics of their otoliths¹⁹. The otoliths of Lake Cazenovia *Lepomis macrochirus* proved to be easily read and unambiguous as to age.

One advantage of using otoliths rather than the more commonly used scales is that with otoliths the annual rings (annuli) can be verified using daily changes in otolith microstructure²⁰. For both female mimics and nesting males, the annuli for the first years of life showed the appropriate number of daily growth increments (E. B. Brothers, personal communication). Although daily increments are more difficult to discern late in life (because of the slowness of growth), the pattern of growth and the appearance of the annuli were unchanged for later years. Daily increments in otolith microstructure have been shown to correspond to daily growth in laboratory reared sunfishes (including *Lepomis macrochirus*), and rings which resembled the annuli of wild caught fish have been induced in laboratory reared *Lepomis cyanellus* by appropriate 'seasonal' temperature manipulations²¹.

Including random population samples, nearly 2,000 fish were aged using otoliths. All otoliths were pooled and read twice, blind with respect to the age, sex and behavioural characteristics of the individual. Using this procedure, female mimics and nesting males were found to show similar age distributions (Fig. 2). Thus female mimics do not represent a normal developmental stage of nesting males. The slightly older age distribution of the nesting males probably results from competition among the males for nesting sites favouring older, larger individuals. Further evidence that female mimics do not mature into nesting males comes from the back-calculated growth rates of the nesting males. If any female mimics were to drastically increase their growth rate and reach nesting male size, the otoliths of some nesting males should show an increase in growth rate late in life. No nesting males showed such a growth increase.

Were alternative behaviour patterns such that individuals could 'switch' from one pattern to another (perhaps better termed 'tactic'), either opportunistically or as a part of development, the genetic substrate for both patterns would be present in every individual. Balancing the relative frequencies of occurrence for these types of alternative patterns is analogous to achieving an optimal mix for the expression of several courtship signals. Each courtship signal need not contribute equally to fitness to be expressed. Demonstrating that female mimics do not exhibit nesting male behaviour, either within a single breeding season or during the course of their ontogeny, however, raises the question of whether this is an example of a genetically maintained, balanced behavioural dimorphism. The next obvious step is to examine the relative contribution of the environment and of the genes to the observed phenotypes.

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At high speeds dolphins save energy by leaping

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An observer may wonder whether a school of 'running' dolphins, consisting of numerous, wildly splashing individuals, is using the most efficient mode of locomotion, because splashing wastes energy. Dolphins exhibit at least three modes of swimming. In leisurely, unhurried motion, they break the surface briefly and gently, often showing little more than the blowhole. At a faster, 'cruising' speed, frequently at 3–3.5 ms⁻¹ (6–7 knots), the animals are seen swimming primarily just beneath the surface, and there is still little splashing. (Behaviour and speeds of dolphin schools were observed from a helicopter and will be described elsewhere by D. A. and W. Perryman.) Swimming speeds in this mode have been measured up to 4.6 ms⁻¹ (9.3 knots). But in the fastest 'running' mode, the animals clear the water in sequential, parabolic leaps, accompanied by considerable splashing on exit and re-entry (Fig. 1). Leaps are interspersed with relatively brief, subsurface swimming. This swimming is common when dolphins are alarmed by vessels approaching within 500 m. We have examined dolphin swimming in terms of energy required per unit distance travelled and report here that beyond a certain 'crossover' speed, leaping must be more efficient than swimming.

The energy cost of leaping must be compared with that of continuous swimming very close to the surface. This is because during rapid swimming, as in escape behaviour, the rate of respiration increases, and the animals are therefore constrained to the surface layer. The extra energy required for jumping is

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approximately (neglecting air resistance)

$$J = WH(1+m) \quad (1)$$

where W is the weight of the dolphin, H is the maximum height of the centre of gravity above the undisturbed sea level, and m is a correction term accounting for the spray carried along with the dolphin as it emerges. The dolphin is approximately neutrally buoyant¹ and so very little energy is required to raise the centre of gravity to surface level. The energy in equation (1) will be compared with that expended by a dolphin swimming continuously with its blowhole just out of the water. This swimming would be roughly equivalent to that of leisurely or cruising swimming, where no more than one-third of the body is out of the water at any instant.

The energy required for swimming at one equivalent body radius beneath the undisturbed surface (that is just submerged) is

$$E = DL \quad (2)$$

where D is the hydrodynamic drag and L is the distance travelled. The drag can be written as²

$$D = \frac{1}{2}\rho_w V^{2/3} C_D \gamma U^2 \quad (3)$$

where ρ_w is the density of water, V is the dolphin volume, C_D the drag coefficient in deep water, γ the correction due to proximity to the surface^{3,4}, and U the swimming speed. Dolphins usually leap only for short periods so that other energy saving aspects of swimming, as when using beat-and-glide or diving⁵, are not applicable.



Fig. 1 A 'running' eastern spinner dolphin (*Stenella longirostris*) leaping approximately two body lengths in the rapid swimming mode. Photograph by R. Pitman.

We now write the ratio of energy J/E to find the range of speeds at which this ratio is <1 , that is leaping is energy sparing. The energies must be compared over the same distance and therefore the length of the jump as a function of swimming speed has to be known. We neglect air resistance. The leaping dolphin has an approximate ballistic trajectory, for which the distance crossed while the centre of gravity of the animal is out of the water is

$$L = \frac{U^2}{g} \sin 2\alpha \quad (4)$$

where g is the gravitational constant and α the emergence angle, measured from the horizon. The maximum height reached by the centre of mass is

$$H = \frac{U^2 \sin^2 \alpha}{2g} \quad (5)$$

The longest leap for given speed U is obtained when $\alpha = 45^\circ$. In this case, designated by subscript 'max',

$$L_{\max} = \frac{U^2}{g}; H_{\max} = \frac{U^2}{4g} \quad (6)$$

Using these results, we obtain the ratio J/E :

$$R = \frac{J}{E} = \frac{\rho_d g V \frac{U^2}{4g} (1+m)}{\frac{1}{2}\rho_w V^{2/3} C_D \gamma \frac{U^2}{g}} = \frac{\rho_d g (1+m) V^{1/3}}{2\rho_w C_D \gamma U^2} \quad (7)$$

where ρ_d is the density of the dolphin. Equation (7) shows that for a given dolphin swimming as described, the ratio R decreases monotonically as forward speed U increases. Thus, for every species, there will be a range of speeds for which leaping will be more efficient than shallow swimming. The lowest (crossover) speed at which this occurs is obtained by setting $R=1$ in equation (7). This speed U_c is dependent on the size of the dolphin (through the volume V), its density, its shape (which will define C_D and m), and how far beneath the surface it swims (which defines γ).

Table 1 Predicted values of crossover speed U_c over which leaping becomes energy sparing as a function of body volume

V (m ³)	U_c (m s ⁻¹)	L_c (m)	H_c (m)	E (J)
0.005	3.34	1.14	0.28	17
0.05	4.90	2.45	0.61	368
0.1	5.50	3.09	0.77	928
0.15	5.89	3.54	0.88	1,590
0.2	6.18	3.90	0.97	2,340
0.25	6.41	4.19	1.05	3,160
1	8.08	6.66	1.67	20,100
5	10.57	11.40	2.85	172,000

Predicted leap length and height are shown, as well as the energy required for each leap ($g = 9.8 \text{ m s}^{-2}$). For any given volume, under-water motion at less than U_c is less energy-consuming than leaping the same distance. A volume of 1 m^3 corresponds to a weight of 1,025 kg.

To obtain specific values of the crossover speed, we substitute typical values of the different parameters in equation (7). The dolphin's density is affected by the degree of lung inflation and the extent of fat reserves. We take it to be approximately equal

to the density of sea water¹, that is, $\frac{\rho_d}{\rho_w} = 1$. The spray mass

coefficient is obtained from the longitudinal added mass, defined as the mass of water carried along when a body moves through a fluid. Thus when the dolphin leaves the water, this mass is carried along. The added mass coefficient for fusiform, elongated shapes such as the dolphin's is $m \approx 0.2$. Hoerner² gives the relationship between the drag coefficient C_D based on volume and skin frictional drag C_f as

$$C_D/C_f = 4(l/d)^{1/3} + 6(d/l)^{1/2} + 24(d/l)^{2/7}$$

which gives a ratio of 8 for a dolphin with an l/d ratio of about 5, where d = diameter and l = length. Hoerner's Fig. 22 shows C_f to be between 0.002 and 0.003 for semi-turbulent flow in the appropriate range of Reynolds number, so that C_D was approximated here as 0.02. The surface effect correction γ is taken as 4.5 when the body is fully submerged, just below the surface. (If the dolphin were an exact body of revolution $\gamma = 5$)^{3,4}. Both C_D and γ should be considered approximations, C_D because it may be a conservative estimate for a flexing body and γ because it is sensitive to submerged depth and decreases rapidly to 1 at a depth three times the body diameter. Substituting all the above values in equation (7) and setting $R=1$, we obtain

$$U_c = 65.3 V^{1/3}$$

where U_c is in m s^{-1} and V in m^3 . This general equation, while only an approximation for any specific dolphin, should give a good estimate of the expected speeds above which leaping will occur. Table 1 gives the values of the crossover speed, U_c , as a function of body volume.

Table 1 indicates that for the usual size range (0.05–0.10 m³) of most dolphin species^{7,8}, the crossover speed is about 5 m s⁻¹ (10 knots), well within their available range of speeds⁹. During January and February 1977 and 1979, the RV David Starr Jordan approached various dolphin species (*Stenella* spp., *Delphinus delphis*) at speeds of 4.5–5 m s⁻¹ (9–10 knots) in the eastern tropical Pacific. At that speed, the vessel could approach close to all schools as long as they could be tracked. However, it often took an hour for the ship to close onto schools initially seen at about 2 miles (3.6 km) distance. Evidently those dolphins were mostly moving at cruising speeds of 3.5–4 m s⁻¹ (7–8 knots), similar to previous measurements. We observed that the leaping (running) mode occurred primarily after the ship had closed to within 500 m and the animals had become increasingly alarmed and had noticeably increased speed. No measurements of actual speed were possible as the leaping dolphins usually escaped abeam and when followed, continued along curved paths. The speeds attained appeared somewhat greater than that of the ship and were probably in excess of the crossover speed. The actual crossover speed is probably within 10% of the predicted value. Thus, because of their shallow swimming ($\gamma \approx 4.5$), the crossover speed must be approximately 5 m s⁻¹ (10 knots), and dolphins must leap to obtain higher speeds efficiently.

Swimming speed can be estimated indirectly by measuring the distance between the emergence spot and the re-entry splash on photographs similar to Fig. 1, and comparing it to animal length. These distances were within the range of 2–4 m, as expected from Table 1. The height of the leaps (to the centre of gravity) is between 0.6 and 1.0 m, also in agreement with Table 1. Spotted and spinner dolphins (*S. attenuata* and *S. longirostris*) have been observed leaping distances of about three body lengths (5.4 m) when chased closely by speedboats, corresponding to a swimming speed of ~ 7.3 m s⁻¹ (14.6 knots)¹⁰. After escaping purse seine nets, they continued leaping for 1,500 m, performing leaps of ~ 2.3 body lengths, which corresponds to a speed of 6.4 m s⁻¹, also greater than the crossover speed.

Larger cetaceans ($V > 1$ m³, weight > 1 ton) probably cannot leap during fast swimming, as the energy required for leaping increases rapidly with body size (Table 1). Fishes, on the other hand, do not have to break surface to breathe and can stay sufficiently deep for surface proximity effects to be negligible ($\gamma = 1$). Crossover speed would then be increased by a factor of more than 2, and, except for large fish such as istiophorids and some large scombrids, is not likely to be an important factor in swimming.

Although the cetacean running mode has been described in the context of escape behaviour, it could occur in any situation where rapid speeds for short periods were required. Furthermore, leaping behaviour is not restricted to the running mode. At any speed, individual dolphins will often 'splash leap', that is leap out of the water with various contortions so as to re-enter with a large splash. The various attitudes of such leaps indicate, however, that they are not primarily related to horizontal velocity. Their function is most probably that of display-communication, the exact meaning depending on the situation, including the physiological state of the animal.

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Role of the blastocoele microenvironment in early mouse embryo differentiation

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During preimplantation development, the mouse embryo differentiates into an outer layer of cells, the trophectoderm, and an inner group, the inner cell mass. Tarkowski and Wróblewska¹ proposed that this differentiation depends on the position of blastomeres in the morula, with outside cells giving rise to trophectoderm and inside cells producing the inner cell mass. This epigenetic hypothesis has been confirmed by other studies in which blastomere position was altered at the 4-cell or 8-cell stage, thus demonstrating the totipotency of the blastomeres at these stages^{2–4}. It was recently found that the inner cell mass of the early blastocyst is also totipotent and can form trophectoderm when isolated by immunosurgery^{5–8}. Because inner cells apparently become committed during the time that they are exposed to the distinct microenvironment of the blastocyst^{9–11}, we postulated that diffusible components or other factors in the blastocoele may have a role in the commitment of cells in the inner cell mass. We report here results, obtained by injecting donor cells into host blastocysts, showing that totipotent cells exposed only to blastocoele fluid differentiate into morphologically normal blastocysts, whereas those in contact with the blastocyst's inner surface do not.

The influence of the blastocoele microenvironment on development of 8-cell and morula-stage embryos was tested by injecting them into the blastocoele of giant chimaeric blastocysts formed by aggregating 8–10 embryos at the 4- to 8-cell stage. These host chimaeras were cultured 48 h, and at the expanded blastocyst stage they were immobilised with a micropositioner and a hole was made in the host trophectoderm with two hand-held glass needles. The zona pellucida of the donor embryo was either kept intact for exposure to diffusible components of blastocoele fluid or removed to allow intimate contact with the inner surface of the host embryo. The donor embryo was inserted into the blastocoele of the host chimaera, which was then allowed to heal. Donor embryos were scored at 24 and 48 h after surgery for escape, arrest, formation of a blastocoele cavity, or formation of a structure without a blastocoele. Some donor embryos were then removed from the host microenvironment by dissection or immunosurgery¹² and tested for their ability to form trophoblast outgrowths *in vitro*.

Host chimaeras readily accommodated the donor embryo within the blastocoele (Fig. 1a), although the host's cavity began to collapse within minutes after an opening was made in the trophectoderm. The procedure required approximately 5 min and was technically difficult to perform on host embryos that were in early stages of expansion. Ten to fifteen host chimaeras were injected in each experiment. In most chimaeras the trophectoderm healed within several hours, as indicated by the reappearance of the host blastocoele cavity.

Two-thirds of the donor embryos that were injected with their zona pellucida intact at the 8-cell or morula stage developed into morphologically normal blastocysts within the host blastocoele (Fig. 1b, c, Table 1). Approximately a fifth of the donors escaped or were unscorable because the host blastocoele failed to reappear, indicating irreparable damage to the host trophectoderm. A few donors arrested at the 8-cell or morula stage, but

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their frequency did not exceed that of control (uninjected) donor embryos (data not shown). After 48 h of culture some donor embryos hatched but remained free floating within the host blastocoele. When these embryos were removed from the host and cultured, they formed outgrowths that had 15–20 trophoblast giant cells like those found in trophoblast outgrowths from normal blastocysts^{13,14}.

Table 1 Injection of zona-intact 8-cell and morula-stage embryos into chimaeric blastocysts*

Stage of donor	No. of donors injected	Fate of donor		
		Unscorable	Arrested	Blastocyst
8-cell	31	8	2	21
Morula	26	2	6	18
Total	57	10 (18%)	8 (15%)	39 (68%)

* Methods as in Fig. 1.

In contrast to the intact donors, only a tenth of the donors that were injected without a zona pellucida developed into blastocysts (Table 2). More than half the donors, however, formed compact structures that were in intimate contact with host cells and lacked a blastocoele (Fig. 2a). Approximately a third of the donors escaped or were otherwise unscorable owing to collapse of the host trophoctoderm; a few donors were arrested at late cleavage stages. The compact structures were generally attached to the inner surface of the host trophoctoderm but occasionally were in contact with the host inner cell mass. To determine whether donor embryos formed compact structures as a result of their trophoctoderm cells becoming integrated into the host, we labelled donor embryos with ³H-thymidine before transfer to the host blastocoele. Our preliminary experiments show that intense autoradiographic labelling was restricted to the compact structure (Fig. 2a, Table 3). Fewer grains were seen in host trophoctoderm cells that were in direct contact with the donor and occasional grains were found in other host cells. We suggest that donor cells were retained within the compact structure and that the host cell labelling may have been due to transfer and reutilisation of thymidine by host trophoctoderm. The thymidine labelling conditions used here (10^{-2} μ Ci ml⁻¹ for 4 h) have been shown not to be toxic to early mouse embryo development¹⁵.

Table 2 Injection of zona-free 8-cell and morula-stage embryos into chimaeric blastocysts*

Stage of donor	No. of donors injected	Fate of donor			
		Unscorable	Arrested	Blastocyst	Compact structure
8-cell	54	23	2	5	24
Morula	80	16	3	10	51
Total	134	39 (29%)	5 (4%)	15 (11%)	75 (56%)

* Methods as in Fig. 2.

To define their developmental potential further we removed the compact structures from host chimaeras 2 days after injection and cultured them in conditions that allow post-implantation development¹⁶. In three experiments, 21 of 27 compact structures derived from zona-free donor morulae formed outgrowths. The outgrowths had 5–15 giant cells, and about half had central clumps of cells resembling inner cell mass derivatives (Fig. 2b). Three compact structures failed to develop, two formed blastocysts, and one formed only an inner cell mass derivative.

We interpret the results of injecting zona-intact donors into chimaeric blastocysts to indicate that diffusible substances that are contained within the blastocoele fluid do not inhibit differentiation of totipotent cells into trophoctoderm. Austin and Lovelock¹⁷ showed that the rat or hamster zona pellucida is readily permeable to 1,200-molecular weight dyes. Thus, all small ions, amino acids, vitamins, hormones and other small metabolites present in blastocoele fluid should pass through the zona. The mouse zona appears to be permeable to at least some high molecular weight substances, because antibodies can affect development^{18,19} and immunosurgery can be carried out with

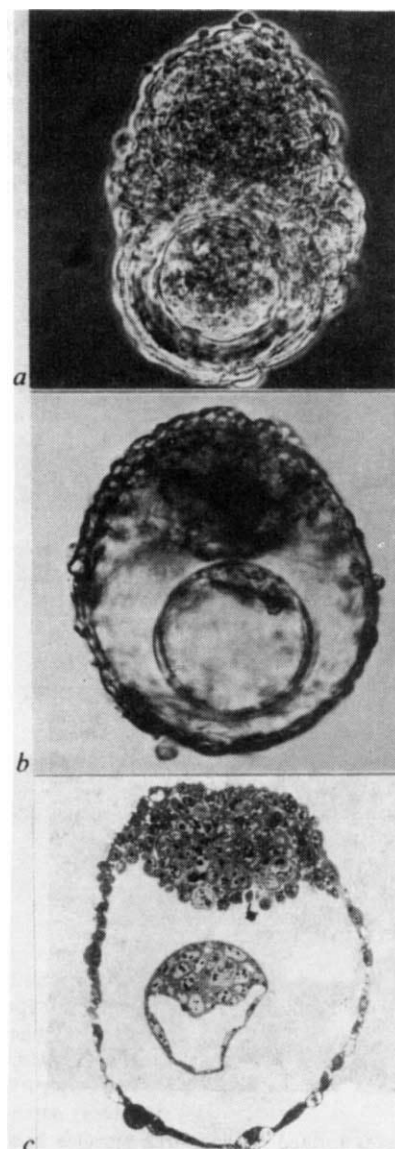


Fig. 1 Chimaeric blastocysts injected with intact donor morulae. *a*, Immediately after injection (phase-contrast, $\times 225$). Donor embryo with intact zona pellucida is in host blastocoele. *b*, After 24-h culture (differential interference contrast, $\times 195$). Donor embryo has developed into morphologically normal blastocyst. *c*, Embryo from *b* fixed and sectioned (bright field, $\times 215$). Embryos were obtained from Dub: (ICR) mice (Flow Laboratories) after superovulation and mating and were cultured in standard egg culture medium as previously described²⁴. Embryos for host chimaeras were obtained on the second day of pregnancy at the 2-cell stage. They were cultured overnight and made into chimaeras by aggregating 8–10 zona-free 4- to 8-cell embryos in phytohaemagglutinin²⁵. Chimaeras were cultured individually in microdrops of medium under a layer of paraffin oil for an additional 3 d. Donor embryos were obtained on the third day of pregnancy at the 8-cell or morula (12- to 16-cell) stage and were inserted into the blastocoele of chimaeras as described in the text. Embryos were fixed and sectioned as previously described¹⁶.

embryos that have an intact zona pellucida¹². As a control, however, we ruptured but did not remove the zonae of eight 8-cell embryos before transfer to host blastocoeles in order to allow free interchange of fluid between the host blastocoele and the donor perivitelline space. Six of the donors developed into blastocysts (one escaped from its zona and formed a compact structure attached to the host trophoctoderm, and one escaped from the host blastocoele). This result shows that even high molecular weight components of the blastocoele fluid do not alter the differentiation of totipotent donor cells.

The results of injecting zona-free embryos into chimaeric blastocysts cannot be interpreted with certainty because we do not yet know the identity of cells in the compact donor structures. Despite their failure to accumulate blastocoele fluid, the compact structures retained the ability to form trophoblast outgrowths when isolated by immunosurgery. In this respect they resembled inner cells isolated from early blastocysts⁵⁻⁸. Contact with host cells may have altered or delayed the differentiation of donor cells into trophoctoderm, or it may have physically prevented them from accumulating blastocoele fluid.

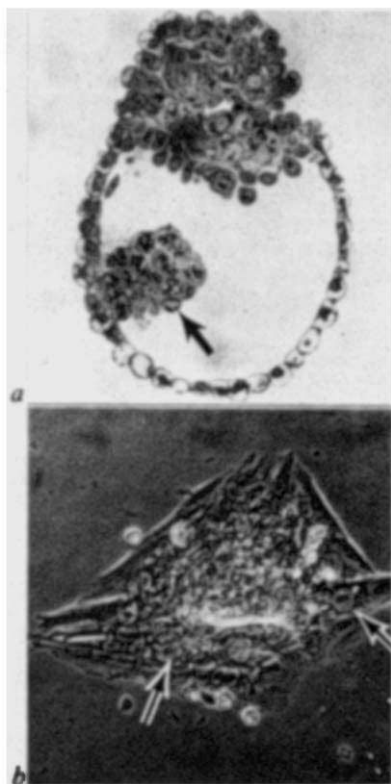


Fig. 2 Structures formed by zona-free morulae injected into chimaeric blastocysts. Host chimaeras were made and donor morulae were injected as described in Fig. 1, except that the donor zona pellucida was removed by a 3–4-min treatment in 0.5% pronase. *a*, Donor morula labelled with ³H-thymidine, injected into host, fixed after 24 h culture, and sectioned (bright field, $\times 240$). Note grains over nuclei of compact structure attached to inner surface of host trophoctoderm (arrow). For labelling of donor cells, morulae were incubated for 4 h in 10^{-2} μ Ci ml⁻¹ ³H-thymidine (specific activity, 40 Ci mmol⁻¹), washed extensively, and incubated in unlabelled medium at least 2 h before transfer to the host blastocoele. For autoradiography, embryos were fixed and sectioned as described previously¹⁶, except that the uranyl acetate treatment was omitted. They were exposed to Kodak NTB emulsion for 2 months. *b*, Outgrowth formed by compact structure (phase-contrast, $\times 128$). After injected chimaeras were cultured 48 h in standard medium, compact structures were removed by immunosurgery and cultured in microdrops of modified Eagle's medium on tissue culture dishes (Falcon) for 6 d by the methods previously described¹².

Table 3 Fate of ³H-thymidine label in donor nuclei*

Location of nuclei	No. of nuclear sections scored	Labelled nuclei (%)	Grains per labelled nucleus (mean $\pm$ s.e.m.)
Compact structure	140	94.3	44.5 $\pm$ 31.5
Trophoctoderm:			
In contact with compact structure	19	89.5	14.3 $\pm$ 11.4
Not in contact with compact structure	286	25.2	2.0 $\pm$ 1.1

* The embryo shown in Fig. 2a was fixed and serially sectioned for autoradiography. Grains were counted over nuclei in 15 sections.

Because morphological features are not adequate for distinguishing between these possibilities, we are currently assessing the biochemical status of the compact structures using two-dimensional protein patterns²⁰ to identify inner cell mass and trophoctoderm. In any case, it seems that intimate contact between totipotent donor embryos and the blastocoele surface of host embryos alters the donors' morphological development. An understanding of the mechanism for this effect may provide clues to the nature of the interaction between inner and outer cells of intact blastocysts. The physical bases of such interactions could include gap junctions²¹, other elements of the cell surface²², or the extracellular matrix²³.

Although we have not excluded the possibility that components of blastocoele fluid can act in concert with non-diffusible components to affect the differentiation of totipotent cells placed in the blastocoele, our results show clearly that blastocoele fluid alone does not alter the early morphological development of totipotent donor embryos. This conclusion is consistent with the fact that trophoctoderm cells are continuously exposed on their interior surfaces to the fluid that they produce in normal blastocysts. Our results further indicate that the mouse trophoctoderm can accumulate fluid even when exposed to the higher concentration of ions present in blastocoele fluid, which is consistent with active ion transport¹¹.

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A new immunodeficiency disorder in humans involving NK cells

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Immunodeficiency disorders have provided much information on the development and interaction of the various B and T lymphoid components in the immune system of man. As the lymphoid system becomes increasingly divided into functional subsets of cells it will be important to find immunodeficiencies affecting newly discovered cell types. Natural killer (NK) cells are a recently described but ill-defined subpopulation of lymphocytes which is thought to play an important part in surveillance against tumour development¹. Mice homozygous for the *beige* gene were found to have a selective deficiency in NK function² and were more susceptible to transplantation of syngeneic tumours as predicted^{3,6}. We report here that patients carrying the analogous, autosomal recessive Chediak-Higashi (CH) gene have a profound defect in their ability to spontaneously lyse various tumour cells *in vitro* by either antibody-dependent or independent mechanisms. Since other cell-mediated cytolytic functions were relatively normal, these results suggest that the *beige* or Chediak-Higashi gene in both man and mouse controls NK function.

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The Chediak-Higashi syndrome in man is a rare, genetically determined disease manifested clinically by abnormal leukocyte granulation, defective pigmentation and an increased susceptibility to infections⁴. Humoral immunity and delayed type hypersensitivity are normal⁵ and children usually die of pyogenic infection, presumably resulting from a host defence abnormality related to defective granules in their polymorphonuclear (PMN) leukocytes^{4,6}. Survivors generally succumb to a lymphoproliferative disorder which may be malignant⁷.

In the present study, two male patients, Lewis R. (age 29 yr) and Larry R. (age 28), siblings from a consanguineous marriage, and eight different age and sex matched normal controls were studied in parallel experiments on three separate occasions over a 1-month period. The detailed clinical history and many previous experiments on these patients have been described elsewhere⁸⁻¹⁰. In the two CH patients the immune status was normal with respect to (1) serum immunoglobulin and complement levels, (2) *in vivo* antibody response to immunisation with *Salmonella typhosa* endotoxin and common vaccines, (3) delayed type hypersensitivity to mumps, *Candida*, dinitrochlorobenzene and SKSD (streptokinase-streptodornase-vidase), and (4) *in vitro* proliferative responses to various lectins such as concanavalin A, phytohaemagglutinin and pokeweed mitogen. Phagocytic function was also normal. At the time of study the patients were free of overt infection, and were not undergoing treatment.

To investigate NK function, peripheral blood was separated by standard Ficoll-Hypaque gradient centrifugation and the mononuclear cell band was selectively depleted of most (>90%) monocytes by adherence to microexudates of detached kidney cell monolayers. As shown in a representative experiment (Fig. 1a) lymphocytes from both CH patients were profoundly depressed in their ability to lyse K562 target cells, a human cell line of myeloid leukaemia origin which is the most sensitive NK target described in the human¹¹. Data pooled from three repeat experiments revealed 85 ± 12 lytic units (LU) per 10^6 cells ($n=6$) for age and sex matched normals and <0.1 LU/ 10^6 ($n=2$) for the CH patients when LU were calculated at 20% lysis. The low response in the CH patients was not altered by

Table 1 The range of cytolytic effector functions of peripheral blood cells from donors with Chediak-Higashi syndrome compared with normals

Effector function	Target cell	Normal controls*		CH donors*	
		Max. lysis†	LU/ 10^6 ‡	Max. lysis	LU/ 10^6
(1) NK (lymphocyte)	Alab	40 (100/1)	40 ± 4	3 (100/1)	<0.01
(2) ADCC (lymphocyte)	Daudi	62 (50/1)	164 ± 30	10 (50/1)	7 ± 2
(3) ADCC (monocyte)	HRBC	43 (100/1)	12 ± 2	43 (100/1)	18 ± 5
(4) ADCC (PMN)	HRBC	60 (100/1)	22 ± 6	59 (100/1)	26 ± 4
(5) Spontaneous (monocyte)	TU5	13 (10/1)	ND	12 (10/1)	ND
(6) Spontaneous (PMN)	K562	34 (20/1)	4 ± 1	30 (20/1)	5 ± 1
(7) Lectin induced (lymphocytes)	P815	73 (5/1)	1,000 ± 80	52 (5/1)	550 ± 320
(8) Lectin induced (PMN)	RBL-5	44 (5/1)	100 ± 6	50 (5/1)	80 ± 25
(9) NK after MLC	K562	33 (40/1)	95 ± 35	1 (40/1)	<0.1

Lines 1, 2: Peripheral blood was separated by centrifugation (1,000g) over Ficoll-Hypaque and the mononuclear cell band was depleted of monocytes by adherence (45 min, 37 °C) to fibronectin microexudates of detached TU5 kidney cells grown to confluence²². Cell loss was approximately 20% in all donors and contamination of the recovered lymphocytes was <1% monocytes as judged by phagocytosis of latex beads or nonspecific esterase staining and <2% polymorphonuclear leukocytes (PMN) was indicated by Wright's stained cytocentrifuge preparations or staining with a specific monoclonal, anti-human PMN antibody (provided by Dr P. Beverley, University College Hospital, London). Lymphocytes were tested in a 4 h ⁵¹Cr release assay against an NK-sensitive target, Alab (breast carcinoma), or an NK-insensitive target, Daudi (IgM bearing EBV lymphoma), pretreated with a 1/50 dilution of rabbit anti-human IgM. Non-antibody treated Daudi cells were lysed 15% by normals and 1% by CH patients at a 50/1 E/T ratio. Lines 3, 4: Mononuclear cells (74% lymphocytes, 23% monocytes) obtained by Ficoll-Hypaque separation consisted of approximately 72% E rosette forming cells (T cells), 10% sIg⁺ cells (B cells) and 27% FcR⁺ cells (IgG) with no significant differences between donors. PMN were obtained by dextran sedimentation of Ficoll-Hypaque pellets and consisted of approximately 80% FcR⁺ cells, 90% of which phagocytosed bovine erythrocytes coated with rabbit anti-bovine erythrocyte antibody (IgG). Effector cells were tested in an 18-h ⁵¹Cr release assay against human erythrocytes (A⁺) pre-coated with a 1/10 dilution of rabbit IgG anti-HRBC antibody. Line 5: Ficoll-Hypaque purified mononuclear cells were incubated on microexudates and adherent cells (4-7%) were recovered by EDTA treatment and vigorous shaking. Effectors (>90% latex⁺, and nonspecific esterase⁺) were tested in a 48 h, ³H-thymidine release assay against TU5 target cells (SV40 transformed mouse kidney cells). Line 6: Cell pellets from Ficoll-Hypaque were sedimented on dextran followed by re-centrifugation on a Ficoll-Hypaque-Percoll mixture²³. Purified populations were 99% PMN as indicated by morphology and staining with monoclonal anti-PMN antibody. Effectors were tested in a 36 h cytostasis assay as measured by inhibition of ³H-TdR uptake during a 12-h pulse. Cytostasis against K562 was not inhibited by catalase²⁴. Lines 7, 8: Lymphocytes or PMN prepared as described above were tested in an 18-h ⁵¹Cr release assay against P815 (mouse mastocytoma) or RBL-5 (Rauscher leukaemia virus induced murine lymphoma) targets in the presence of 2 or 20 µg/ml⁻¹ phytohaemagglutinin (PHA) respectively. No lysis occurred in the absence of PHA and these concentrations were not toxic. The predetermined optimum PHA concentrations were identical in careful titration experiments, between normals and CH patients. Line 9: Mononuclear cells were cultured 7 d together with a pool of irradiated stimulator cells pooled from four normal allogeneic donors as described previously²⁴. Effectors were collected and tested in a 4-h ⁵¹Cr release assay against K562 cells, an HLA negative target. Lysis by non-stimulated cultures was approximately half the values shown.

* Two CH patients (Le.R., La.R.) and 2-6 normal, age and sex matched controls were studied in parallel experiments.

† Mean % lysis in triplicate wells at the effector/target ratio given in brackets. These values occurred on the plateau of effector titration curves.

‡ A lytic unit was defined as the number of effector cells required for 20% lysis. ND, not determined.

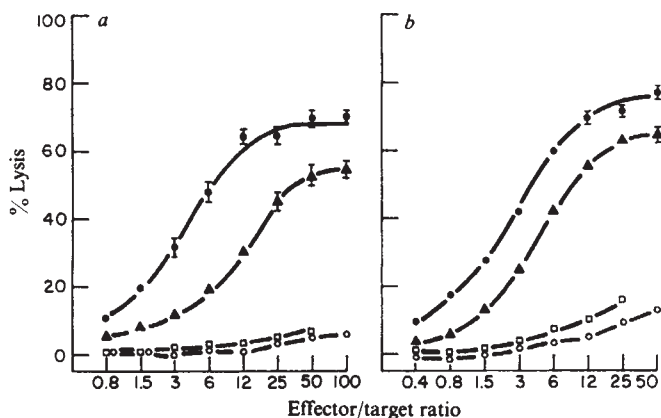


Fig. 1 Impairment of NK and ADCC activity in lymphocytes from Chediak-Higashi (CH) patients. Ficoll-Hypaque separated, peripheral blood lymphocytes were depleted of monocytes on microexudates and incubated with ^{51}Cr -labelled K562 cells, a human myeloid leukemia cell line (a), or antibody-coated Chang cells, a human hepatocarcinoma cell line (b) at various effector-to-target ratios in a 4-h cytolytic ^{51}Cr release assay. ●, J.R.; ▲, J.O. (normal donors); □, La.R.; ○, Le.R. (CH patients). Values represent the mean % specific lysis $\pm$ s.e. in triplicate wells. Lysis of non-antibody coated Chang cells at a 50/1 effector/target ratio was 18, 7, 2, and 1.5% for J.R., J.O., La.R. and Le.R., respectively.

prolonged incubation time (up to 24 h) and was evident in tests of six different NK-sensitive target cell lines including MOLT-4, Alab, CEM, MDA and human fetal fibroblasts (data not shown). The NK defect persisted during a 7-d incubation *in vitro* with or without stimulator cells (Table 1, line 9). As shown in Fig. 1b, lysis of Chang cells, precoated with an optimum dose of rabbit anti-Chang antibody, was also severely depressed. The degree of cytolytic depression was similar against antibody-coated Daudi targets, a human lymphoblastoid cell line (Table 1, line 2) and lysis of antibody-coated RBL-5, a murine tumour, was also markedly impaired although less so than the response against human tumours (unpublished observation). The failure of CH lymphocytes to efficiently lyse human tumours cells was not due to a generalised block in the antibody-dependent cell-mediated cytotoxicity (ADCC) mechanism. Hence mononuclear effector cells and polymorphonuclear leukocytes (PMN) from CH patients lysed antibody-coated human erythrocytes in a normal fashion as shown in Table 1 (lines 3, 4). When combined with the cell fractionation studies shown in Table 2, these results suggest

Table 2 Fractionation of NK and ADCC effectors

Expt	Fractionation	% Lysis of K562*		% ADCC*	
		Normal	CH	Normal	CH
1	FcR ⁺	29	4	36	3
	FcR ⁻	6	0.2	1	0
2	Unseparated	51(14)	5(<1)	59(97)	10(<1)
	G-10 passed	53(53)	1(<1)	64(133)	22(4)
3	Unseparated	22(9)	2(<1)		
	Nylon passed	39(11)	0.4(<1)		
4	Microexudate	60(50)	7(0.3)		
	Microexudate + interferon	75(100)	14(4.0)		

Expt 1: Peripheral blood mononuclear cells were depleted of monocytes by a 1-h incubation on plastic surfaces. Lymphocytes were then incubated 60 min at room temperature in plastic flasks (Falcon) pre-coated with trinitrophenylated (TNP) fetal calf serum (FCS) and rabbit anti-TNP antibody²⁵. Non-adherent lymphocytes (FcR⁻) were recovered in the supernatant and adherent cells (FcR⁺) were eluted with EDTA and scraping. FcR⁺ and FcR⁻ fractions were incubated overnight at 37 °C to allow recovery before assay. **Expt 2:** Mononuclear cells were passed through a 10-ml column of Sephadex G-10 equilibrated with medium containing 20% FCS. Cell recovery was 60% of input. **Expt 3:** Mononuclear cells were passed through a 10-ml column packed with scrubbed nylon wool (Fenwall). Cell recovery was 50% of input. **Expt 4:** Mononuclear cells were depleted of monocytes on microexudates and preincubated for 1 h with 10^3 units ml^{-1} human fibroblast interferon (HEM Research) or medium as control, washed and assayed for cytotoxicity.

* Effectors were assayed in a 4-h ^{51}Cr release assay against K562 cells or RBL-5 cells pretreated with rabbit anti-mouse thymocyte serum. Values represent the mean % lysis in triplicate wells at a 50/1 E/T ratio and the numbers in brackets represent lytic units per 10^6 calculated at 20% lysis.

that the defect in ADCC and NK in CH patients is confined to a subpopulation of FcR⁺, non-adherent lymphocytes selectively blocked in their ability to lyse tumour cell targets.

The selectivity of the antibody-independent NK cell defect is shown in Table 1. Lymphocytes were blocked in their ability to spontaneously lyse tumour cell targets whereas cytolysis of tumour cells by monocytes was normal (line 5). Surprisingly, cytostasis by PMN from CH patients against K562 cells was also normal (line 6), as was lectin-generated killing by this cell type (line 8). Since lysosomal enzymes and degranulation are abnormal in CH patients (reviewed in refs 4, 6) these results suggest that lysosomal granules are not involved in PMN cytostasis or cytolysis against these target cell lines. The population of T killer cells, as measured by lectin-dependent cytolysis was normal in one patient (Le) but low in the other (La, line 7). A more extensive study is required before a definitive statement on the status of cytolytic T cells can be made. However, T-cell mediated immunity in general was previously shown to be normal in these and other patients as judged by skin testing and proliferative responses to T-dependent mitogens⁸⁻¹⁰.

Table 3 Frequency of target binding cells

Donor	Fractionation	% TBC		
		K562	Molt-4	P815
Normal	Nylon wool passed	24 $\pm$ 4*	37 $\pm$ 3	2 $\pm$ 1
CH	Nylon wool passed	25 $\pm$ 2	36 $\pm$ 4	3 $\pm$ 2
Normal	G-10 passed	15 $\pm$ 2	37 $\pm$ 3	ND
CH	G-10 passed	15 $\pm$ 1	43 $\pm$ 5	ND

Ficoll-Hypaque separated peripheral blood lymphocytes were passed over nylon wool or Sephadex G-10 columns to remove adherent cells and mixed with a fivefold excess of tumour cells. Cell mixtures were centrifuged (150g) 5 min, incubated for 5 min at 37 °C and then stored on ice. Pellets were gently resuspended and the number of lymphocytes binding to tumour cells (TBC) was determined in triplicate samples by size discrimination in a haemocytometer as described previously¹³.

* Values represent the mean $\pm$ variance of %TBC values for two individuals. Nylon wool passed or G-10 passed cells from normals yielded 55%, 46% and 1% lysis of K562, Molt-4 and P815 respectively in a 4-h ^{51}Cr release assay, at a 100/1 E/T ratio. Lysis by CH cells was below 5%. ND, not determined.

Further experiments suggested that the CH defect may lie within the lytic pathway rather than at the level of population size. As shown in Table 3, non-adherent lymphocytes from CH patients were completely normal in their ability to bind and lyse K562 and Molt-4 targets, whereas P815, an NK-insensitive target, was not bound or lysed by CH or normal donors. Most of the lymphocytes binding to tumour cells (TBC) detected in this system have been shown to represent NK cells in the mouse¹² and in the human TBC can be specifically inhibited by pre-incubation of effector cells with solubilised glycoproteins from NK-sensitive targets¹³. Therefore, if the relative number of TBC in a heterogeneous human lymphocyte population is a reliable estimate of the frequency of NK cells, as in the mouse, then these results suggest that NK cells are present in CH patients but do not function.

Suppressor cells in the NK system appear primarily to be adherent, macrophage-like cells (reviewed in ref. 14). Suppressor cells did not appear to be responsible for the low NK response of CH patients as removal of FcR⁻ cells or cells adherent to Sephadex G-10, nylon, wool or microexudates did not restore the low NK response in CH patients (Table 2). In addition no suppression of cytolysis was seen in mixtures of CH and normal lymphocytes, even at the highest ratios tested (2/1, data not shown).

The precise location of the defective gene product in the cytolytic pathway is not known but may involve an impairment of cyclic GMP-mediated triggering of NK cells as: (1) cyclic GMP causes a small but significant enhancement of NK activity in mice whereas cyclic AMP decreases the level of cytolysis¹⁵; (2) cyclic GMP or inducers thereof have been shown to improve various defects in CH patients such as abnormal granule

morphology¹⁶, chemotaxis¹⁷ and NK function (P. Katz, unpublished observation), and (3) interferon, which boosts NK activity in CH patients (Table 2) is known to cause a transient increase in cyclic GMP levels in lymphoid cells¹⁸.

Thus we have described the first immunodeficiency disorder in humans with an apparently selective deficit in effector functions mediated by NK cells and cells involved in ADCC. Other studies of the more familiar immunodeficiencies, involving impairments of both B and T cells, failed to reveal significant defects in NK function¹⁹ except in the case of severe combined immunodeficiency²⁰. Impairments of ADCC are more widespread (reviewed in ref. 19) and in one study of X-linked agammaglobulinaemia, ADCC was defective whereas NK activity was normal²⁰. In view of the complexity involved it is not surprising that different genes should control unique steps in an otherwise common cytolytic pathway in a single cell, possibly mediating both ADCC and NK activity as previously suggested (reviewed in ref. 21).

It is intriguing to note that 85% of the 53 CH cases on record entered an accelerated 'lymphoma-like' phase¹⁰. Closer scrutiny of this phenomenon may provide the first direct evidence that NK cells in the human are involved in surveillance against spontaneous tumour development.

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Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation

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In near-physiological concentrations, glucocorticoid hormones cause the death of several types of normal and neoplastic lymphoid cell, but the mechanisms involved are unknown^{1,2}. One of the earliest structural changes in the dying cell is widespread chromatin condensation, of the type characteristic of apoptosis, the mode of death frequently observed where cell deletion seems to be 'programmed'^{3,4}. It is shown here that this morphological change is closely associated with excision of nucleosome chains from nuclear chromatin, apparently through activation of an intracellular, but non-lysosomal, endonuclease.

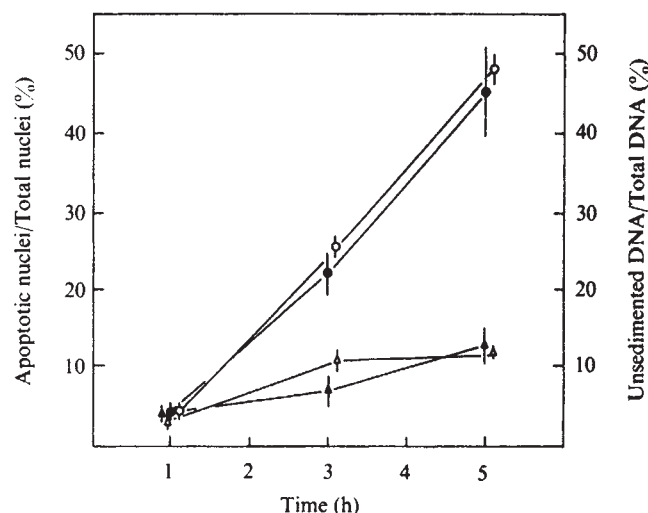


Fig. 1 In methylprednisolone-treated thymocytes the proportion of chromatin cleaved endogenously to small fragments is closely similar to the proportion of nuclei whose morphology shows the generalised chromatin condensation of apoptosis. The graph shows the percentage of total chromatin unsedimented by centrifugation at 27,000g for 20 min in lysates prepared from thymocytes incubated *in vitro* for the times shown, in the presence (●) or absence (▲) of 10^{-5} M methylprednisolone succinate. Plotted on the same axis is the proportion of nuclei in matched cultures showing the morphology of apoptosis after incubation with (○) or without (△) 10^{-5} M methylprednisolone. Steroid concentrations down to 10^{-7} M gave similar results. All points are means of at least five experiments ± 1 s.e. Cells for chromatin studies were washed free of steroid and growth medium by gentle centrifugation (150g for 10 min) and resuspended in 150 mmol l⁻¹ NaCl, pH 7.0. Aliquots of 600 μ l containing 10^8 cells each were lysed in 10 vol of 25 mmol l⁻¹ acetate buffer, pH 6.6. The lysates were centrifuged at 27,000g for 20 min and acid-precipitable DNA was measured by the diphenylamine reaction¹⁶ in the supernatant, the pellet and aliquots of the original uncentrifuged lysate. Recovery from the 27,000g pellet plus supernatant was consistent at over 82% of the original lysate, and incubation of the lysate at pH 6.6 for up to 20 min at 37 °C did not materially alter the proportion of DNA appearing in the 27,000g supernatant. This proportion is expressed as percentage of the total in the uncentrifuged lysate. Cells for morphological study were centrifuged at 150g for 10 min, resuspended in a drop of serum, smeared on glass slides, air dried, fixed in Bouin's fluid and stained by the Feulgen reaction. In each experiment 500 cells were scored for the presence of condensed or normal chromatin.

Suspensions of thymocytes from suckling rats were treated *in vitro* with methylprednisolone succinate for various periods. Thereafter, they were studied by light microscopy to determine the proportion of nuclei with condensed chromatin; matched samples were lysed for measurement of the proportion of chromatin DNA which did not pellet after centrifugation at 27,000g for 20 min. In untreated fresh thymocytes this proportion was near zero, but in treated thymocytes the unsedimented low molecular weight DNA represented an increasing proportion of the total, rising above control levels after 1 h of treatment (Fig. 1). This proportion showed a striking quantitative identity to the proportion of nuclei found to contain condensed chromatin morphologically. The proportion of condensed chromatin also rose progressively with time of exposure to steroid in the treated thymocytes but remained low in the controls (Fig. 1).

Agarose gel electrophoresis showed this low molecular weight DNA to consist of fragments whose molecular weights were integer multiples of a subunit; the length of this subunit—about 180 base pairs—was identical to that of nucleosomes isolated from whole fresh thymocyte nuclei after micrococcal nuclease digestion (Fig. 2). One result of steroid action seemed to be excision from nuclear chromatin of nucleosome chains up to more than 8 units in length. This nucleosome excision was not merely the result of the process of lysis itself, but seemed to have

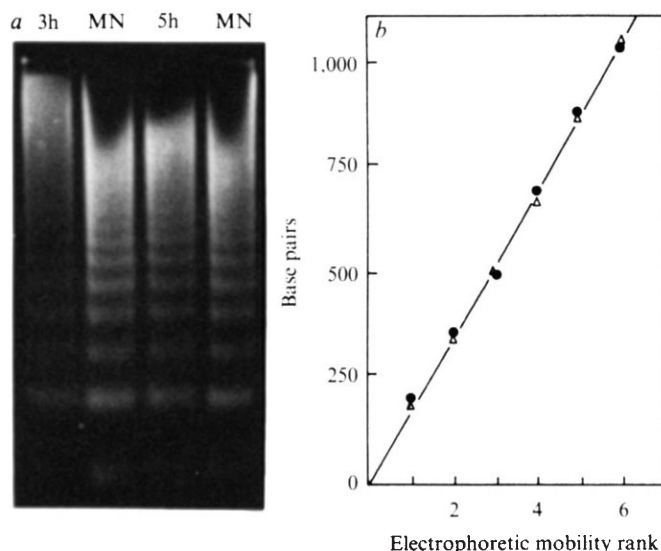


Fig. 2 The low molecular weight chromatin recovered from steroid-treated thymocytes consists of nucleosome chains with a normal repeat length. *a*, Shows a 1.8% agarose gel stained with ethidium bromide and viewed in UV light. The tracks were loaded with DNA from the 27,000g supernatants of lysates of equal numbers of thymocytes after incubation for 3 or 5 h, as indicated, in the presence of 10^{-5} M methylprednisolone succinate, or, for comparison, with a micrococcal nuclease digest of fresh thymocyte nuclei (MN). Thymocyte DNA was concentrated by precipitation in 70% ethanol and 0.13 mol l^{-1} NaCl, dried under vacuum, redissolved in Tris 10 mmol l^{-1} , EDTA 1 mmol l^{-1} and 1% SDS, pH 7.4, and extracted four times through chloroform:isoamyl alcohol (24:1). *b*, Shows the size of the DNA fragments obtained from thymocytes treated for 5 h with methylprednisolone 10^{-5} M (●) and from fresh thymocyte nuclei digested with micrococcal nuclease (Δ). Mobilities in 1.8% agarose were calibrated against a *Taq* digest of ΦX174.

occurred within intact cells, as untreated cells showed little or no such excision, whereas treated cells showed excision after lysis by several different non-shearing methods, including the use of cation chelators and SDS.

These results show that glucocorticoid induces in thymocytes an endonuclease-like activity whereby well organised chromatin is subjected to multiple double-strand cleavage events, restricted to the inter-nucleosomal DNA. Moreover, the close quantitative identity between the appearance of morphologically condensed chromatin and the products of chromatin digestion implies that this activity may be present only in cells showing the characteristic generalised chromatin condensation of apoptosis. We do not know whether the chromatin excision is itself responsible for the condensation.

The closely delineated size distribution of the excised DNA fragments suggests that the endogenous digestion involves nuclease activity without concurrent protease activity. This is evidence against the view that the endogenous, steroid-associated digestion is merely the result of lysosomal activation. Histochemistry of apoptotic cells also suggests that lysosomal disruption does not occur^{4,5}.

In conclusion, the results demonstrate the presence within glucocorticoid-treated thymocytes of new endonuclease activity which excises well organised nucleosome chains from chromatin. This activity becomes evident after about 1 h of glucocorticoid treatment and correlates quantitatively with the morphologically observed chromatin condensation of apoptosis. Lysosomal enzymes are apparently not involved.

As inter-nucleosomal chromatin breaks can be produced by non-enzymatic means⁶, the evidence that this steroid-associated activity is enzymatic is at present presumptive. However, nucleases capable of mediating this type of chromatin excision are known to be present in the nuclei of thymocytes⁷ and other cells^{8,9}, and inhibitors of macromolecular synthesis prevent chromatin condensation in steroid-treated thymocytes⁴. One

hypothesis, which we are now testing, is that divalent cations mediate the lethal glucocorticoid effects. It is well established in other systems that divalent cations promote chromatin condensation¹⁰, and are required for nuclease activation^{8,9}. Further, certain manifestations of lethal glucocorticoid action on thymocytes have been shown to be cation dependent^{11,12}, although these manifestations were observed many hours later than the chromatin condensation and nuclease activation recorded here. Internucleosomal chromatin breakage has been recorded in lymphocytes and fibroblasts during death induced by immunological attack (although apparently not during lysis as a result of viral infection)¹³, and also in the terminal differentiation of normoblasts¹⁴ and lens cells¹⁵. This raises the question of whether a class of endonuclease exists whose activation is associated with the programmed destruction of the genome.

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Insulin-like stimulation of glucose oxidation in rat adipocytes by vanadyl (IV) ions

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The mechanism of insulin action is still unknown¹. One approach to this problem is to apply substances which mimic the action of the hormone to target cells. Ouabain and deprivation of extracellular K^+ (refs 2, 3), which inhibit the active transport of Na^+ and K^+ ions, are both known to activate glucose transport and oxidation in isolated adipocytes. Vanadate (V) ions have recently been shown to act as very efficient inhibitors of the sodium pump or $(Na^+ + K^+)ATPase$ in *in vitro* preparations⁴. They have a natriuretic and diuretic effect in rats⁵ and a positive inotropic effect on cat heart muscle⁶. Many tissues contain vanadium at a concentration of about 0.1–1.0 μM (ref. 7) and so endogenous vanadate could be a physiological regulator of the sodium pump. But this is still open to debate, because the bulk of the vanadium is probably in the vanadyl (IV) form^{8,9} and VO^{2+} ions bind tightly to proteins^{8,10}. VO^{2+} is a relatively ineffective inhibitor of $(Na^+ + K^+)ATPase$ *in vitro*^{8,11}. We report here that externally applied vanadate ions at low concentrations mimic fully the effect of insulin on glucose oxidation in rat adipocytes. However, this simulation seems to be due mainly to the effects of vanadyl (IV) ions, probably produced within the cells, and not primarily to inhibition of the sodium pump. Also, externally applied vanadyl (IV) ions stimulate glucose oxidation substan-

tially. Vanadyl ions are known to be powerful inhibitors of alkaline phosphatase¹² and we therefore consider the possibility that they inhibit a cellular phosphatase activity. An early event in insulin action may involve alteration of the degree of phosphorylation of protein(s) involved in regulation of sugar transport.

The basic effects of insulin and vanadate on glucose oxidation in adipocytes are summarised in Table 1. There is evidence that in adipocytes the rate of oxidation is limited by the entry of glucose into the cell^{13,14}. Insulin alone routinely stimulated the rate of glucose oxidation by three- to fourfold, and an optimal concentration of vanadate ions in the medium (see Table 1 and Fig. 2) normally produced a 70–100% maximal activation of glucose oxidation. As reported previously¹⁶ externally applied ATP partially inhibited the effect of insulin, and this is also the case with vanadate (Table 1). Also, a non-hydrolysable analogue adenosine 5'-(β , γ -imido)triphosphate (AMP-PNP) was ineffective, as found before with insulin¹⁶. Further evidence for the similarity of the effects of vanadate and insulin is the identical stimulation of glucose oxidation over a wide range of glucose concentrations. The degree of stimulation falls as the glucose concentration in the medium is raised, suggesting that the site of activation is the saturable glucose transport system. Removal of Ca^{2+} from the medium did not affect stimulation of the glucose oxidation by either insulin or vanadate (data not shown).

Table 1 Stimulation of glucose oxidation in fat cells by insulin and vanadate. Effects of ATP and AMP-PNP

Addition	¹⁴ CO ₂ production* (c.p.m. per 2 h $\pm$ s.e.m.)	% Maximal stimulation
None	5,800 $\pm$ 200	0
Insulin (10 ng ml ⁻¹)	24,500 $\pm$ 400	100
Insulin (10 ng ml ⁻¹) + ATP (0.4 mM)	15,000 $\pm$ 100	48
Vanadate† (0.1 mM)	23,000 $\pm$ 220	90
Vanadate (0.1 mM) + ATP (0.2 mM)	18,700 $\pm$ 240	68
Vanadate (0.1 mM) + ATP (1.6 mM)	13,600 $\pm$ 200	41
Vanadate (0.1 mM) + AMP- PNP (0.1 mM)	23,200 $\pm$ 140	92

Isolated fat cells were prepared from Sprague-Dawley rats (80–120 g) according to Rodbell¹⁵. Glucose oxidation was measured by conversion of D-[U-¹⁴C]glucose to ¹⁴CO₂ (ref. 15). The final reaction volume of 0.5 ml contained 0.16 mM glucose and about 3×10^5 fat cells, suspended in Krebs-Ringer buffer (pH 7.4) consisting of: NaCl, 110 mM; NaHCO₃, 25 mM; KCl, 5 mM; KH₂PO₄, 1.2 mM; CaCl₂, 1.3 mM; MgSO₄, 1.3 mM and bovine serum albumin, 7 mg ml⁻¹.

* The absolute rate of glucose oxidation with insulin was 4.8 nmol per 2 h. The assay was run in duplicates.

† Sodium orthovanadate (Na₃VO₄) was applied.

Inhibition of (Na⁺ + K⁺)ATPase and active Na and K transport in red cells¹⁷ and squid axons¹⁸ by vanadate ions has been shown to require the presence of K⁺ in the medium. This was clearly not the case for stimulation of glucose oxidation by externally applied vanadate (see Table 2). Removal of K⁺ from the medium inhibits sodium pump activity and gave a partial stimulation of glucose oxidation as described previously². But addition of vanadate in the K⁺-free medium gave a further near maximal stimulation. In fact the vanadate was somewhat more effective in the K⁺-free than in the normal K⁺-containing medium (compare lines 3 and 6 of each experiment, Table 2).

The data in Fig. 2 provide evidence that the principal form of vanadium involved in stimulation of glucose oxidation is the vanadyl ion. The $K_{0.5}$ for externally applied ortho vanadate was about 20 μ M. Addition to the medium of the reducing agent glutathione (GSH) (see refs 4, 11) lowered the concentration of added vanadate for a half maximal stimulation by almost one order of magnitude ($K_{0.5} \approx 3 \mu$ M). GSH (1.2 mM) itself

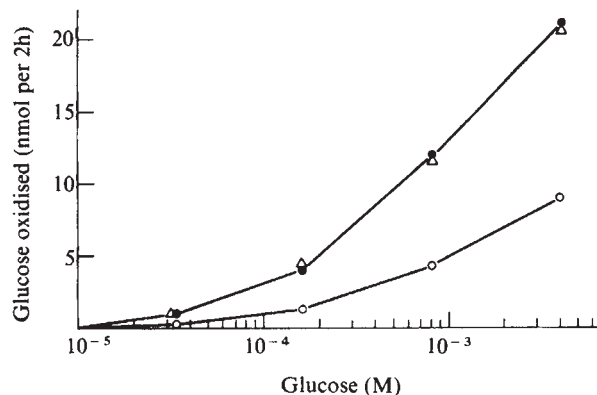


Fig. 1 Rates of glucose oxidation with insulin and vanadate at increasing concentrations of glucose. Isolated fat cells (about 3×10^5 cells) were incubated for 2 h at 37°C in the conditions described in Table 1, except that the D-[U-¹⁴C]glucose was diluted with unlabelled D-glucose to give the final concentrations indicated. Samples containing either insulin (10 ng ml⁻¹, ●), Na₃VO₄ (0.2 mM, △) or no addition (○) were run in duplicate.

produced a small but significant stimulation of the rate of glucose oxidation in these cells (5–15% of the rate of saturating insulin concentrations)¹. Adrenaline (200 μ M) also increased the effectiveness of suboptimal concentrations of vanadate (not shown). Addition of vanadyl ions directly to the incubation medium produced a substantial but incomplete stimulation of glucose oxidation, with an apparent $K_{0.5}$ of $\sim 25 \mu$ M. The presence of GSH did not affect maximal stimulation or the apparent affinity for this effect of VO²⁺. Control experiments showed that solutions of vanadyl sulphate above 100 μ M at neutral pH become cloudy, and so the lack of full stimulation is probably attributable to limited solubility of VOSO₄.

In red cells externally applied vanadate is transported into the cell on the anion carrier¹⁰, and is reduced largely to the vanadyl ion (IV) which itself is bound tightly to haemoglobin⁸. In adipocytes also, preliminary measurements using the vanadyl (IV) ESR signal, indicate that externally added vanadate or vanadyl ions penetrate into the cells and vanadate (V) is reduced to the vanadyl (IV) state by the endogenous reducing compounds (H. Degani, Y.S. and S.K., unpublished data). Both internally generated vanadyl ions and externally applied vanadyl ions become bound to intracellular components (H. Degani, Y.S. and S.K., unpublished). Glutathione is known to be lost from many cells when they are incubated in a GSH-free medium¹⁹. Thus externally applied GSH (see Fig. 2) may enter the adipocytes and supplement the endogenous reducing power, shifting the ratio of vanadate to vanadyl in the direction of vanadyl. The higher concentrations of VO²⁺ required, compared to vanadate plus GSH could reflect slow penetration or binding of VO²⁺ to

Table 2 Stimulation by vanadate ions of glucose oxidation in a K⁺-free medium

Expt	Condition	¹⁴ CO ₂ production (c.p.m. $\pm$ s.e.m.)
26979	K ⁺ -containing medium	7,775 $\pm$ 426
	+ insulin (4 ng ml ⁻¹)	23,200 $\pm$ 201
	+ Vanadate (100 μ M)	14,850 $\pm$ 652
	K ⁺ free medium	17,000 $\pm$ 1500
241079	+ Insulin (4 ng ml ⁻¹)	23,850 $\pm$ 50
	+ Vanadate (100 μ M)	22,875 $\pm$ 727
	K ⁺ containing medium	5,650 $\pm$ 351
	+ Insulin (10 ng ml ⁻¹)	20,100 $\pm$ 500
	+ Vanadate (100 μ M)	14,425 $\pm$ 20
	K ⁺ -free medium	7,700 $\pm$ 201
	+ Insulin (10 ng ml ⁻¹)	19,650 $\pm$ 952
	+ Vanadate (100 μ M)	16,400 $\pm$ 301

Glucose oxidation was carried out as described in the legend to Table 1. In the K⁺-free medium KCl was omitted and NaH₂PO₄ replaced KH₂PO₄.

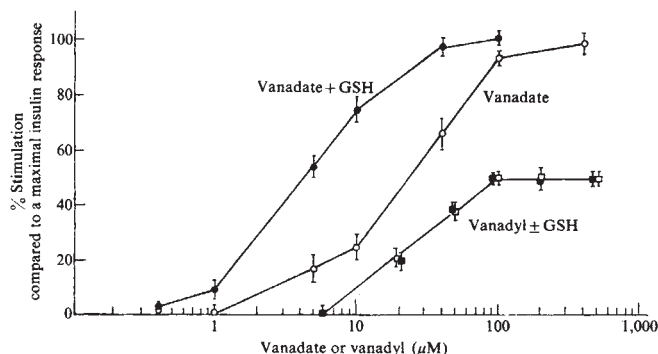


Fig. 2 Rates of glucose oxidation at 0.14 mM glucose were measured as in Table 1. In this experiment maximal stimulation by insulin was 445% of the control value. Where indicated reduced glutathione was present at a concentration of 1.2 mM. The small stimulation of glucose oxidation by GSH alone was subtracted from that observed in the presence of vanadate or vanadyl ions.

the albumen in the medium. Specific ^{125}I -insulin binding to the adipocytes was not affected by vanadate (not shown).

In the light of these results it is unlikely that added vanadate acts primarily by inhibiting $(\text{Na}^+ + \text{K}^+)\text{ATPase}$. The vanadate ion is known to affect several enzymes involved with phosphate or phosphate substrate metabolism²⁰. However, the effective species in our experiments seems to be the vanadyl ion which is known to inhibit strongly alkaline phosphatase¹². If in the adipocytes the vanadyl ions inhibited a phosphatase activity this could imply a connection between the degree of phosphorylation of cellular proteins (the glucose carrier itself?) and regulation of glucose entry by insulin or vanadate. Insulin has been shown to stimulate phosphorylation of certain proteins in adipocytes equilibrated with orthophosphate^{21,22}. The steady state level of phosphorylation should reflect the balance between the relevant phosphorylating and phosphatase activities. Insulin action could be accompanied by inhibition of a phosphatase activity as proposed for added vanadate or vanadyl ions. Alternatively the hormone action may involve stimulation of phosphorylating enzymes. Inhibition of the $(\text{Na}^+ + \text{K}^+)\text{ATPase}$ by removal of external K^+ or by ouabain or vanadate, should save cellular ATP, making more available for phosphorylation.

Vanadium is found in many cells, including classical target tissues for insulin such as skeletal muscle and liver. If, as seems likely, it is mainly in the vanadyl form and at physiological concentrations of 0.1–1.0 μM , it is available for interaction with cellular phosphatases, it could play an important part in controlling protein phosphorylation *in vivo*. An attractive possibility is that insulin bound to the cell exterior alters either the ratio of vanadyl to vanadate concentrations, or binding of the VO^{2+} ion to cytoplasmic proteins, that is VO^{2+} could be a second messenger. Possible functions of internal vanadyl ions are being investigated.

As far as we are aware this work represents the first demonstration of an effect by vanadyl ions on an important physiological process in whole cells. Whether this finding has wider implications for regulation of cytoplasmic and membrane processes by vanadyl ions remains to be established.

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A site for the potentiation of GABA-mediated responses by benzodiazepines

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The benzodiazepines have been well characterised as minor tranquillizers and attempts to explain their unique spectrum of activity have included suggestions that they may interact with a variety of neurotransmitter systems¹. Recently, a possible interaction with the γ -aminobutyric acid (GABA) system has received most attention. Benzodiazepines potentiate the actions of both synaptically released and exogenously administered GABA^{2–4} on mammalian neuronal preparations but the site of action within the GABA response mechanism has not been determined. Binding studies suggest that benzodiazepines combine with highly specific sites in the neuronal membrane⁵ and that these sites have some indirect association with GABA receptors^{6–11}. To investigate this association further in a functioning GABA system, quantitative studies have been made *in vitro* on neuronal depolarisations mediated by GABA receptor activation. Evidence has already been presented¹² that bicuculline is most probably a competitive antagonist at the GABA receptor while picrotoxin acts as an antagonist at a separate site. Here flurazepam is shown to attenuate preferentially the action of picrotoxin rather than bicuculline and a model is suggested for the site of action of these drugs within the GABA response mechanism.

Slices of rat cuneate nucleus were prepared as described previously¹³ and arranged in a two-compartment bath such that drugs could be superfused over the terminal regions of the afferent fibres to the nucleus and consequent changes in membrane polarisation could be recorded¹². Muscimol (Fluka) was used routinely as the agonist for GABA receptors in these quantitative studies to avoid the complications which arise when agonists such as GABA are rapidly removed by a saturable uptake process¹². Muscimol depolarised the nerve fibres in a dose-dependent manner. In the presence of 10^{-6} M flurazepam-HCl (Roche), the dose-response curve was usually shifted to the left in a parallel fashion (Fig. 1). This potentiation of muscimol was not very large but, in replicate experiments, it proved to be significant with a mean muscimol dose ratio of 0.808. At a higher concentration of flurazepam, 10^{-5} M, there was no further increase in the potentiation of muscimol and at the lower concentration of 10^{-7} M there was no significant potentiation at all.

With flurazepam still present, either (+)bicuculline (Sigma) or picrotoxin (Sigma) were added to the superfusion fluid¹² and the lower part of the muscimol dose-response curve was re-determined. Both antagonists displaced the dose-response curve to the right in a parallel fashion. For each concentration of antagonist, the equipotent muscimol dose ratio was determined and the results are presented in the form of Schild plots in Fig. 2. Control data obtained from similar experiments in the absence

of flurazepam¹² are also shown. As there was no way of determining whether bicuculline and picrotoxin antagonised the potentiating effect of flurazepam as well as antagonising muscimol, the results have been calculated in two ways: (1) assuming no antagonism of the potentiation *per se* and (2) assuming complete antagonism of the potentiation at all concentrations of antagonist. When assumption (1) was made, the potency of bicuculline as an antagonist of muscimol was not significantly reduced by flurazepam 10^{-6} M. The potency of picrotoxin, however, was consistently reduced, 2.67 times as much picrotoxin being required to achieve the same degree of antagonism as in the control experiments. When assumption (2) was made, there was an extra small reduction in the potency of both antagonists but the overall reduction in picrotoxin potency was still considerably greater than the reduction in bicuculline potency. An increase in the concentration of flurazepam to 10^{-5} M caused no further reduction in the potency of picrotoxin and flurazepam 10^{-7} M was ineffective. Other benzodiazepines must be tested to establish whether the observations with flurazepam reflect a general property of this class of drug.

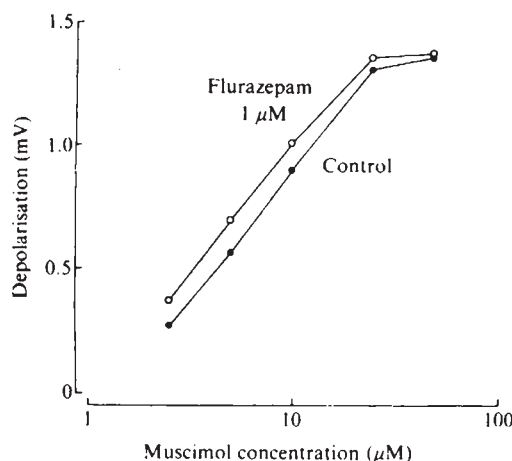


Fig. 1 Depolarisations of afferent nerve fibres in a slice of rat cuneate nucleus by 2 min superfusions of muscimol. Peak responses are plotted against log muscimol concentration before (●) and during (○) the superfusion of flurazepam 10^{-6} M. Each point is a single response. The equipotent muscimol dose ratio in this experiment was 0.771. In replicate experiments, the equipotent muscimol dose ratio (mean $\pm$ s.e.m.) in the presence of flurazepam 10^{-6} M was 0.808 ± 0.047 ($n = 24$) which is significantly less than 1 ($P < 0.01$); in the presence of flurazepam 10^{-5} M it was 0.837 ± 0.086 ($n = 12$) and in flurazepam 10^{-7} M it was 0.905 ± 0.047 ($n = 9$). In this, as in other experiments, flurazepam had little direct effect, the most usual response being a small and transient depolarisation. In control experiments, there was no sign of sensitisation or tachyphylaxis upon repeated administration of muscimol.

Interpretation of these results in terms of the site of action of flurazepam depends largely on a knowledge of the sites of action of the GABA antagonists bicuculline and picrotoxin. In the preparation used here, and in other neuronal preparations from vertebrate species, the evidence points to bicuculline being a competitive antagonist at the GABA receptor while picrotoxin acts at a different site¹². This distinction is corroborated by the demonstration of separate binding sites for bicuculline and picrotoxin^{14,15}. Penicillin appears to act as a GABA antagonist at yet another site which may be the chloride ion channel opened by the GABA receptor¹⁶. A hypothetical model of the GABA response mechanism incorporating these different sites is shown in Fig. 3.

The site of action suggested for flurazepam is based on the observation of a preferential reduction in the potency of picrotoxin rather than bicuculline and an assumption of a single site for benzodiazepine action. It seems unlikely that flurazepam and picrotoxin competed for a common site since increasing the concentration of flurazepam did not result in a further reduction

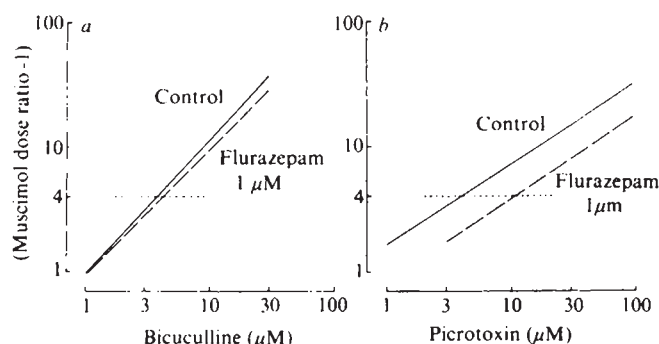


Fig. 2 Schild plots of the antagonism of muscimol by bicuculline (a) and picrotoxin (b). log (muscimol dose ratio - 1) is plotted against log (antagonist concentration) and each line was calculated by least squares regression analysis from 18–28 points. The continuous lines represent antagonist potency in the absence of flurazepam¹². The broken lines represent antagonist potency in the presence of flurazepam 10^{-6} M, the dose ratios being obtained by comparison of the muscimol dose-response curves in the presence of flurazepam and flurazepam + antagonist. The slopes of the bicuculline lines were: continuous 1.070 ± 0.032 , broken line 1.008 ± 0.070 . The slopes of the picrotoxin lines were: continuous line 0.635 ± 0.021 , broken line 0.655 ± 0.049 . Comparisons of the intercepts of the lines²⁰ at the level (muscimol dose ratio - 1) = 4 indicated: a, no significant difference between the bicuculline lines ($P < 0.1 > 0.05$) at an equipotent bicuculline concentration ratio of 1.11 b, a significant ($P < 0.001$) reduction in the potency of picrotoxin in the presence of flurazepam 10^{-6} M at an equipotent picrotoxin concentration ratio of 2.67. These comparisons of antagonist potency are based on an assumption that bicuculline and picrotoxin did not antagonise the potentiating action of flurazepam (assumption (1) in the text). Re-calculation of the muscimol dose ratios by comparing control dose-response curves with those obtained in the presence of flurazepam + antagonist (assumption (2) in the text) resulted in a small additional displacement to the right of the Schild plots obtained in the presence of flurazepam. The lines remained parallel to those obtained in the absence of flurazepam and the equipotent antagonist concentration ratios increased to 1.57 for bicuculline and 3.80 for picrotoxin.

in the potency of picrotoxin. The data from binding studies also generally indicate that benzodiazepines and picrotoxin bind to separate sites^{8–11,15} although it has recently been found that benzodiazepines can reduce the binding of dihydropicrotoxin to brain membranes¹⁷. In Fig. 3, therefore, flurazepam is suggested to potentiate an endogenous process which facilitates or prolongs the opening of the ion channel and with which picrotoxin competes. Such an action of flurazepam could account for the small potentiation of muscimol. A change in the properties of the GABA receptor⁶ appears to be a less likely explanation since the potency of the GABA receptor antagonist bicuculline was little affected by flurazepam. The model does not

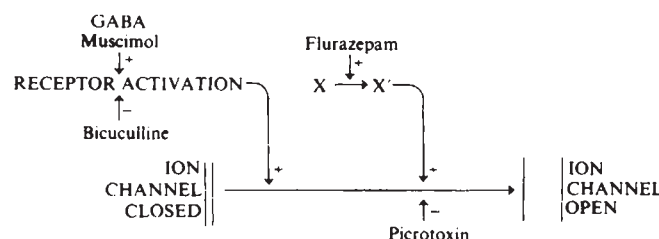


Fig. 3 Hypothetical model of the GABA response mechanism and the possible sites of action of some drugs. +, Agonist or facilitatory action, including antagonism of an inhibitory influence; -, indicates antagonism. $X \rightarrow X'$ represents an endogenous process which facilitates or prolongs channel opening. The intermediate steps between receptor activation and ion channel opening seem to be the minimum necessary to accommodate the pharmacological data from depolarisation responses to GABA and muscimol of afferent nerve fibres in the rat cuneate nucleus.

allow for an enhancement of benzodiazepine binding by GABA receptor activation⁸⁻¹¹, but there is now a possibility that this effect may involve a different population of GABA receptors from those mediating changes in membrane conductance and polarisation^{18,19}.

This model has been devised as the simplest arrangement which would accommodate all the drug effects on responses to GABA receptor activation reported here and which would also be compatible with the data from binding studies. Although the stages between GABA receptor activation and ion-channel opening are entirely hypothetical, they do have possible implications. The model suggests either (i) that flurazepam increases the duration of channel opening for each GABA receptor activation or (ii) that only a proportion of the GABA receptor activations result in channel opening and that flurazepam increases that proportion. It also seems that there is no requirement for a fixed ratio of benzodiazepine sites to GABA receptors in the neuronal membrane. It remains to be seen which of these predictions are correct, but it is clear that responses to GABA receptor activation can be modulated at a number of different sites.

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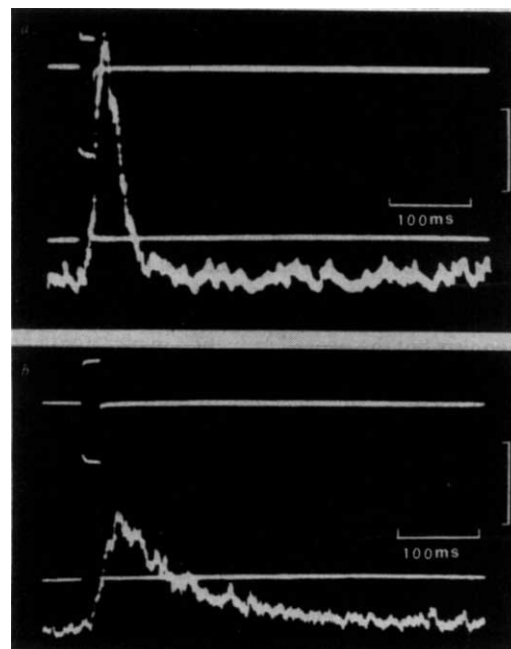


Fig. 1 Calcium transient in extensor digitorum longus (a) and (b) soleus muscle fibres of rat. The lowest traces show the aequorin responses, the middle ones record membrane potentials and the upper ones the clamp currents. Calibration bars are 2 nA for the light responses, 50 mV for the membrane potentials and 1 μ A for the clamp current. Temperature, 25 °C. A time constant of 5 ms was used for the light recordings.

with two bevelled microelectrodes. One was filled with aequorin, dissolved in 120 mM KCl, 25 μ M EDTA, 5 mM HEPES buffer (pH 7.9), and was used both for injecting aequorin into the fibre and for recording membrane potential. The other, inserted at a distance of 50-150 μ m, was filled with either 3 M KCl or 4 M K-acetate and was used to pass current across the muscle fibre membrane to clamp the membrane potential, which was normally held at -80 mV. Aequorin was injected, under microscopic inspection by applying a pulse of pressure to the aequorin pipette. The light emitted, when Ca^{2+} combines with aequorin⁸, was recorded with a photomultiplier (EMI 9824) run at -900 V and placed about 1.5 cm above the surface of the muscle. Usually, the EDL and Sol were both mounted in the same chamber to standardise the experimental conditions.

When the muscle fibre membrane was strongly depolarised, a flash of light was recorded (Fig. 1), indicating a rise in the level of ionised calcium in the myoplasm. The Ca^{2+} is presumably released from the sarcoplasmic reticulum, since similar light responses could be recorded in Ca^{2+} -free solutions with 1 mM EGTA. Applying pulses of 20-60 ms duration, and graded intensity, to fibres held at -80 mV, light was first detected when the membrane potential was stepped to about -35 mV. This level was not very different from that at which a localised contraction in both fast and slow muscle fibres was first detected. However, in some cases the threshold depolarisation for light detection was higher than that for contraction, and the maximum light response was smaller. This probably occurred when the amount of aequorin injected by the pressure pulse was very small, or when some of the aequorin contained in the tip of the micropipette had been inactivated by the external Ca^{2+} , before insertion into the fibre. The amplitude of the light response with increasing depolarisations rose sharply at first and then tended to level off. Increasing the pulse duration increased the rise time, and amplitude of the light response. Thus, in many respects, the relationship between membrane potential and light response in mammalian muscles fibres resembles that obtained previously with arsenazo III⁶ and with aequorin (our unpublished data) in frog muscle fibres.

Calcium transients in mammalian muscles

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Contraction of vertebrate skeletal muscle is caused by calcium ions released from the sarcoplasmic reticulum (see refs 1, 2 for reviews). The ensuing transient change in the intracellular level of ionised calcium has been monitored using various Ca^{2+} indicators, such as murexide,³ aequorin^{4,5}, and arsenazo III^{6,7}. So far, most of what is known about these calcium transient derives from experiments on barnacle or frog muscle fibres, and it is desirable to extend such studies to mammalian muscle. We report here that the photoprotein aequorin^{8,9} can be used to monitor calcium transients in rat and human muscles, and that the transients decay more quickly in fast contracting muscle fibres.

The experiments were done on the fast-twitch extensor digitorum longus (EDL) and slow-twitch soleus (Sol) muscles of the rat, and on human intercostal muscle obtained during thoracic surgery. The isolated muscles were mounted in a bath perfused with oxygenated mammalian Ringer¹⁰, which contained $1-3 \times 10^{-7}$ g ml⁻¹ tetrodotoxin to abolish action potentials, and in most experiments 10 mM tetraethylammonium bromide was used to reduce delayed rectification. Muscle fibres were impaled

With depolarising pulse durations of a few milliseconds to 100 ms, the light response increased during the pulse and continued to rise well after the membrane potential had returned to the holding level (Fig. 1). A continued rise of the aequorin signal is also seen after the termination of iontophoretic pulses of Ca^{2+} discharged into droplets of aequorin¹¹. This suggests that the time during which calcium is released from the sarcoplasmic reticulum is shorter than the time to peak of the light response, and probably not much longer than the voltage clamp pulse. Presumably the depolarising pulse opens Ca^{2+} channels in the membrane of the sarcoplasmic reticulum and these channels, like those at motor nerve terminals¹², are rapidly closed once the membrane is repolarised.

The rate of rise and peak amplitude of the light response greatly depends on the amount of aequorin injected into the fibre. This makes it difficult to compare responses obtained in different muscle fibres, because it is difficult to load them always to the same degree. Nonetheless, it seems that the light response is greater in the fast EDL muscle than in the slow soleus. Preliminary examination of the relationship between membrane potential reached by the depolarising pulse, and the amplitude of the ensuing light response, indicates that the relationship is S-shaped in both fast and slow muscle fibres. The threshold for light detection is roughly the same for both types of muscles fibres, but thereafter the amplitude of the light responses to depolarising steps of increasing intensity rises more slowly, and attains a lower maximum, in the soleus fibres.

After reaching its peak, the light response decayed nearly exponentially, with a time constant that was essentially independent of the intensity and duration of the depolarising pulse. In analogy with previous results⁷ obtained in frog muscle, there was a marked difference in the rate of decay of the light responses of fast and slow muscle fibres of the rat. At 25 °C the decay time constant in EDL fibres was 16.0 ± 4.0 ms (mean $\pm$ s.d. of 35 fibres from 14 muscles), while in Sol fibres, it was 42.4 ± 13 ms (mean $\pm$ s.d. of 65 fibres from 13 muscles). With application of longer pulses, the light response decayed during the pulse. This decay was much slower than that after the end of a short pulse and is presumably caused by inactivation of the Ca^{2+} release mechanism from the sarcoplasmic reticulum, although other factors such as depletion of Ca^{2+} stores may be also involved. Again, the light in EDL fibres decayed more rapidly than in the soleus. The duration from half rise to half decay was 244 ± 64.6 ms (mean $\pm$ s.d. of 8 fibres), while in Sol fibres it was 468 ± 118 ms (mean $\pm$ s.d. of 16 fibres). The greater dispersion in the rate of decay of Ca^{2+} transients in soleus muscle fibres, may be related to the existence, in this muscle, of slow and 'intermediate' (10%) motor units¹³; but so far our results are inadequate to establish whether there are two populations of muscle fibres with clearly distinct Ca^{2+} transients. In both fast and slow muscles the light responses became slower with decreasing temperature. In the range between 15 and 40 °C, the time constant of decay changed more or less linearly with inverse temperature in Arrhenius plots in both muscles and the Q_{10} was

2.4 for EDL and 2.0 for Sol, but the difference of the Q_{10} value between EDL and Sol may not be significant considering the range of the values obtained at each temperature.

Calcium transients were also recorded from human intercostal muscle fibres; but their time course in the two samples examined so far was found to be very different. For instance, in one muscle (Fig. 2) the decay time constant of the aequorin light was 20.4 ± 3.0 ms (five fibres) while in a muscle from a different subject, it was 108 ± 22 ms (nine fibres). At present we do not know the cause of such a large difference.

The rate of rise and peak amplitude of the calcium transients in rat EDL muscle fibres was generally greater than in the soleus. This suggests that the rate of release of calcium from the sarcoplasmic reticulum may be greater in the fast fibres. Such a difference could arise from a corresponding difference in the number, or kinetic characteristics of the Ca^{2+} releasing sites in the reticulum of fast and slow fibres. The decay of the light responses reflects the time course of the processes which restore the intracellular calcium ion concentration to its resting level. It is not yet clear to what extent uptake of calcium by the sarcoplasmic reticulum is involved in determining the decay of the light responses. However, the marked difference in the rate of decay of light responses in EDL and Sol muscles is apparently in line with the observation that the sarcoplasmic reticulum is less developed^{14,15} and, when isolated, takes up calcium more slowly^{16,17} in the soleus muscle.

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Progressive inhibition of the Ca pump and Ca:Ca exchange in sickle red cells

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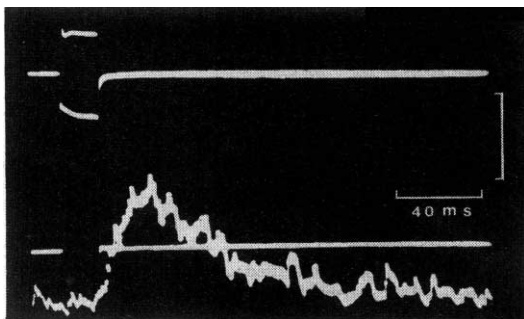


Fig. 2 Calcium transient in human intercostal muscle. Traces as Fig. 1. The calibration bar represents 2 nA for the light response, 50 mV for the potential and 1 μ A for the current. Temperature, 25 °C. Time constant of light recording system, 5 ms.

Sickle cell anaemia red cells (SS) were reported to have a high Ca content and an increased Ca uptake on deoxygenation^{1,2}, but their Ca-pump activity was described as normal². This seemed puzzling because the saturated Ca-extrusion rate of the normal, high Ca-affinity Ca pump is about 10 mmol per 1 cells per h (refs 3, 4) and the highest sickling-induced Ca influx reported in SS cells^{2,5} and observed in ATP-depleted sickle-trait (SA) red cells⁶ never exceeded 0.2 mmol per 1 cells per h. Normal pump performance is, therefore, incompatible with Ca accumulation unless SS cells have abnormally high Ca-binding capacity. We provide here evidence which suggests that SS cells have normal Ca-buffering capacity and probably genetically normal Ca pumps, but that the sickling process causes progressive Ca-pump failure and a marked reduction in Ca:Ca exchange.

Table 1 Cell-associated calcium pools in SS and normal (AA) red cells—total, ionophore mobilisable (free) and ^{45}Ca -tracer exchangeable in 2.5 h

Method	Conditions	Estimated calcium content of cells (μmol per 1 cells)	
		SS cells	AA cells
Atomic absorption spectroscopy	Washed in EGTA-free buffer	52.0 ± 8.2 (4)	11.6 ± 1.4 (3)
	Washed in EGTA-buffer	40.3 ± 17.2 (11)	5.7 ± 1.8 (7)
	After incubation with ionophore and EGTA	5.8 ± 2.3 (5)	4.7, 5.4 (2)
^{45}Ca influx	Washed in EGTA-buffer	4.5 ± 1.6 (5)	1.3, 2.0 (2)
	After incubation with ionophore and EGTA	0.26 ± 0.04 (4)	<0.20 (1)

For atomic absorption spectroscopy, red cells from fresh heparinised blood were washed four times with an ice-cold medium containing (in mM): NaCl, 140; KCl, 5; HEPES, 10 (pH 7.40); with or without EGTA, 0.1, and all buffy coat removed. Some samples were exposed to ionophore (5 μM) in the presence of EGTA at 37 °C for 15 min before the final washes. After washing, the cells were lysed in distilled water, and haemoglobin precipitated with 5% trichloroacetic acid (TCA). Distilled water was deionised and gave a signal equivalent to less than 0.05 μM Ca. All the buffers used in the analysis were passed through Chelex 100 resin, TCA was redistilled following Harrison and Long¹⁵ and all plastic tubes and tips were rinsed with 5% redistilled TCA and deionised water before use. Atomic absorption spectroscopy was carried out on a Perkin-Elmer Model 360 spectrophotometer with an HGA 2100 graphite furnace, having a sensitivity of 15 pg Ca, and permitting accurate ($\pm 5\%$) measurement of <0.5 μM Ca. Ca values (mean $\pm$ 1 s.d.) are expressed in $\mu\text{mol l}^{-1}$ red cells, assuming a mean corpuscular haemoglobin concentration of 34 g dl^{-1} for the cells. The number of samples, each from a different patient, is shown in parentheses. Increasing the EGTA in the wash buffer from 0.1 to 5 mM did not lower the levels of red cell Ca by atomic absorption spectroscopy. The ^{45}Ca -tracer exchangeable calcium pool was estimated by measuring the influx of ^{45}Ca . Washed cells were resuspended at 10% haematocrit in the EGTA-free washing medium containing, in addition, 10 μM glucose and 1 mM $^{45}\text{CaCl}_2$, and incubated for 2.5 h at 37 °C. Subsequent washing and measurement of ^{45}Ca in the red cells were as described by Ferreira and V.L.L.⁸. The ^{45}Ca content of the cells was calculated by dividing the radioactivity per unit haemoglobin-estimated cell volume by the external specific activity of ^{45}Ca .

We first investigated the cytoplasmic Ca buffering of intact SS cells by the method of Ferreira and Lew^{3,7}. The equilibrium distribution of ^{45}Ca between cells and medium induced by a high concentration (10 μM) of the ionophore A23187 was measured with red cell suspensions (haematocrit $\sim 10\%$) from four sickle cell anaemia patients. The medium (A) contained (mM): NaCl, 75; KCl, 75; MgCl_2 , 0.2; HEPES-Na (pH 7.4), 10; glucose, 10, and two different initial concentrations of $^{45}\text{CaCl}_2$, of 0.020 and 0.100. At equilibrium, the SS cells contained 6.8–10.2 times more ^{45}Ca than equal volumes of suspension medium. The fraction of ionised Ca calculated from these values was 0.20–

0.29, falling within the lower part of the normal range (0.20–0.45)³.

It was important to determine whether the high original cell-associated Ca of the SS cells was free to equilibrate with added ^{45}Ca in the presence of the ionophore, so that the equilibrium distribution of the tracer would reflect the true distribution of the free Ca. Table 1 shows that addition of the ionophore A 23187 to EGTA-washed SS cells suspended in EGTA-containing media removed 86% of the cell-associated Ca. This indicated that most of the original cell-associated Ca was intracellular and free, and that it was available for equilibration with added ^{45}Ca in the presence of the ionophore.

There was a residual level of tightly bound, ionophore-resistant and non-exchangeable cell Ca, of about 5 μmol per 1 cells, associated with both SS and normal cells. The surprising observation, however, was that in the absence of the ionophore, only about 10% of the free, ionophore-mobilisable Ca of SS cells exchanged with ^{45}Ca in 2.5 h, whereas all of the free, ionophore-mobilisable Ca of AA cells had equilibrated with the ^{45}Ca -tracer in that time. The tracer-equilibration rate of the SS cells is therefore at least 20 times slower than that observed in ATP-depleted AA cells⁸, the only conditions in which similar-size Ca pools can be maintained inside normal intact cells without ionophore or Ca-pump interference. The conclusion that Ca:Ca exchange must be extremely slow in at least some of the Ca-containing SS cells is further supported by efflux measurements from ^{45}Ca -preloaded SS cells. We see (Table 2, condition 4) a progressive reduction in the efflux of ^{45}Ca into a medium containing 1 mM ^{40}Ca which, even after correcting for net ^{45}Ca release (from values in Table 2, condition 3), suggests a marked heterogeneity of exchange rates. After 2 h, the efflux of ^{45}Ca through all pathways combined had become negligibly small, whereas the cells still retained almost 22% of the initial ^{45}Ca pool. Furthermore, as there was no net Ca gain in these conditions (see Table 2, condition 3), all Ca movement across the membrane of those cells containing the residual ^{45}Ca pool, whether through pump, leak or Ca:Ca exchange, had virtually ceased.

We investigated Ca-pump function by following the release of ^{45}Ca from SS cells preincubated in the presence of 1 mM $^{45}\text{CaCl}_2$ in the various experimental conditions described in Table 2. It can be seen (Table 2, conditions 2 and 3) that ^{45}Ca efflux is uphill, because it proceeds unchanged in the absence or presence of a large inward Ca gradient. The efflux, however, is two orders of magnitude smaller than that expected from a normal, high Ca-affinity pump saturated with internal Ca and ATP. As the ATP concentration per unit volume of cell water is apparently normal in SS cells, even in the dense cell fraction rich in irreversibly sickled cells⁹ whose Ca levels are particularly

Table 2 ^{45}Ca release from ^{45}Ca -preloaded SS cells

Condition	Pretreatment during ⁴⁵ Ca loading	Final incubation medium	⁴⁵ Ca content of cells at start of final incubation	⁴⁵ Ca efflux	Period during which flux measured (min)
			(μmol per 1 cells)	(μmol per 1 cells per h)	
1	Aerated, 90–180 min	Ca-free, 1 mM EGTA	2.9, 6.0 (2)*	0.6, 1.3	0–70
2	Deoxygenation, 90–180 min	Ca-free, 1 mM EGTA	19.2, 27.6 (2)	19.9, 20.6	0–10
				4.9, 5.7	10–70
3	Deoxygenation, 120 min	1 mM ⁴⁵ CaCl ₂	15.8 (1)	16.0	0–12
				3.6	12–50
4	Deoxygenation, 120 min	1 mM ⁴⁰ CaCl ₂	17.6 (1)	22.8	0–20
				8.7	20–40
				5.0	40–60
				2.1	60–90
				0.76	90–120

Red cells from fresh, heparinised blood were washed and resuspended (haematocrit, about 15%) in a medium similar to that used to measure cytoplasmic Ca buffering (medium A, see text) but containing 1 mM $^{45}\text{CaCl}_2$. The cell suspensions were incubated under air (aerated) or 100% argon (deoxygenation) for the indicated times. After four washes in an ice-cold, Ca-free medium, the cells were resuspended in medium A at about 10% haematocrit and incubated at 37 °C, under air, for up to 2 h. The presence in the suspension medium of EGTA, ^{40}Ca or ^{45}Ca of the same specific activity as that used during the loading period was as indicated. The ^{45}Ca content of the cells was calculated by dividing the ^{45}Ca activity per unit cell volume by the external specific activity of ^{45}Ca ; this calculation underestimates the true Ca content of the cells, because not all cell Ca had equilibrated with the tracer (see Table 1). It seems, however, a convenient way of expressing the results because it conveys some information on the approximate magnitude of the true Ca fluxes. The residual ^{45}Ca content of the cells in condition 4 after 2 h was 4.1 μmol per 1 cells. Conditions 3 and 4 report the ^{45}Ca loss from the same cells in strictly comparable conditions.

* Mean values in each experiment. Number of experiments in parentheses.

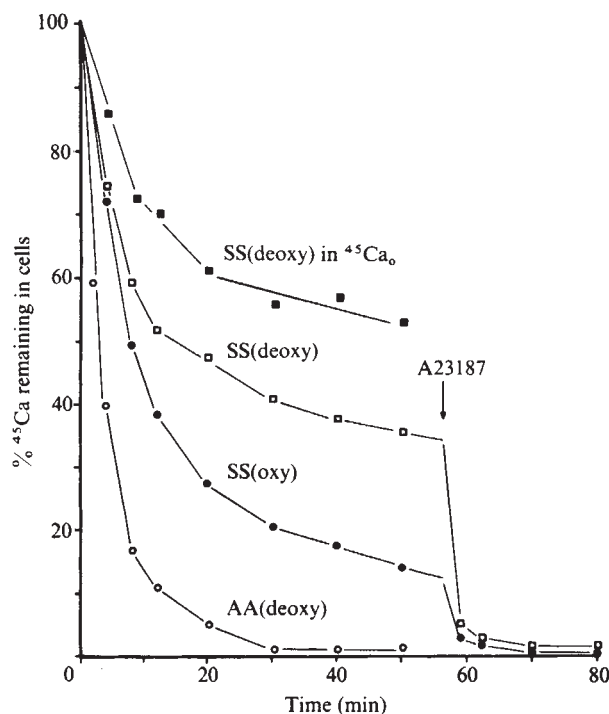


Fig. 1 Effect of a deoxygenation pulse on the percentual release of ^{45}Ca from normal (AA) and sickle (SS) red cells preloaded with ^{45}Ca in the presence of the ionophore A23187. Red cells from fresh heparinised blood were washed and resuspended at about 15% haematocrit in a medium of similar composition to that used to measure cytoplasmic Ca buffering (see text) but containing 1 mM $^{45}\text{CaCl}_2$. After 90 min incubation under air (oxy) or argon (deoxy), the ionophore A23187 was added to give a final concentration of 1 μM in the cell suspension, and the incubation continued for 90 s. The cells were then washed and resuspended at an haematocrit of about 10% in the same medium. In addition, each suspension also contained EGTA, 1 mM except for the one group noted to contain $^{45}\text{CaCl}_2$, 1 mM, with the same specific activity as that used during the loading period. The residual ^{45}Ca content of the cells was measured in the TCA supernatant of the washed cell lysate⁸. Ionophore (1 μM) was added after 55 min in two of the conditions with EGTA-containing media to show that the slowly extruded Ca pool had been retained by a permeability barrier, and was not tightly bound to cell components but was free to diffuse out of the cells. Comparison of the SS (deoxy) efflux curves in the absence and presence of ^{45}Ca shows that ^{45}Ca efflux represents uphill extrusion against a steep inward Ca gradient. The small difference between the two curves represents the full magnitude of the inward Ca leaks, including any residual ionophore-mediated permeability. The contribution these leaks could have made to the observed ^{45}Ca efflux would, therefore, have been unmeasurably small. The initial ^{45}Ca content of the cells at the beginning of the final incubation and designated 100% in the curves shown was (in μmol per 1 cells): AA (deoxy), 436; SS (oxy), 187; SS (deoxy), 74.6. In a similar experiment, not shown here, the ^{45}Ca content of SS (oxy) cells fell from 51.6 to 5.8 μmol and per 1 cells in 45 min and that of SS (deoxy) cells fell from 35.1 to 12.9 μmol per 1 cells in the same period. The percentual changes were almost identical in the two experiments despite the different initial ^{45}Ca -content values.

high^{1,2}, the low Ca-extrusion rate must reflect an abnormal response of the Ca pump itself.

The nature of this Ca-pump failure was investigated by experiments similar to those described in Table 2, except that 60–90 s before the end of the preincubation the cells were more uniformly loaded with ^{45}Ca by a brief exposure to the ionophore A23187. After washing away the ionophore^{10,11}, the release of ^{45}Ca was investigated as shown in Fig. 1. We see that the ionophore allowed ^{45}Ca entry into a population of SS cells with normal Ca pumps, for the initial extrusion rate was similar in SS and normal cells. In contrast to normal red cells, however, a substantial fraction of the SS cell population exhibited weak Ca-pump activity. Furthermore, following a sickling pulse, either the fraction of weak pumping cells or the size of the Ca pool inside these cells, or both, seemed to be substantially increased.

Because some of the rapidly extruded Ca must have come from cells that had been sickled during the preincubation, a single sickling episode is not always sufficient for Ca-pump

inhibition. The mechanism of inhibition is unknown but it could involve irreversible dissociation of calmodulin from the inner pump surface, as observed with the $(\text{Mg}^{2+} + \text{Ca}^{2+})\text{ATPase}$ of SS ghosts¹². Inaccessibility of calmodulin, and even Ca, to their receptor sites on the Ca pump might result from a shielding effect by the large amount of membrane-bound, denatured haemoglobin S which has been observed in those ghosts^{13,14}.

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Nitrogen-fixing growth by nonheterocystous cyanobacterium *Plectonema boryanum*

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Many oxygen-evolving nonheterocystous cyanobacteria can synthesise nitrogenase^{1,2}, but only a few are able to protect the enzyme from inhibition and denaturation by oxygen³⁻⁵. It is difficult to reconcile concomitant oxygenic photosynthesis and the obligately anaerobic process of nitrogen fixation in the remaining strains. One possibility is a temporal separation of the processes: *Plectonema boryanum* (U. Tex strain 594) has been shown⁶ to fix nitrogen and then grow oxygenically to the limits of the fixed nitrogen when incubated without combined nitrogen under N_2/CO_2 . Growth was not extensive. Here conditions are described in which the same strain of *P. boryanum* will simultaneously fix nitrogen and grow extensively, fully pigmented, apparently with sufficient metabolic energy to drive nitrogen fixation.

Preformed or exogenous carbohydrate is necessary for the expression of nitrogenase in strains such as *P. boryanum* when the enzyme is induced under N_2/CO_2 (ref. 2). Some cyanobacteria seem to prefer anoxic, reducing environments⁷, and some strains are capable of anoxygenic photosynthesis, oxidising hydrogen sulphide to sulphur⁸. *P. boryanum* is incapable of anoxygenic CO_2 fixation⁹, may be able to oxidise hydrogen sulphide to sulphur¹⁰, and will grow heterotrophically¹¹. It seemed that added carbohydrate might substitute for the lack of CO_2 fixation and that reducing conditions might inhibit oxygenic photosynthesis⁸ or chemically reduce free oxygen and thus enable nitrogen fixation and growth to occur simultaneously. Colony growth was obtained on fructose-supplemented plates

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incubated in an anaerobic jar into which an atmosphere of 95% $N_2/5\%$ CO_2 was introduced through repeated partial evacuation, and then sulphide was added by injecting an Na_2S solution. The jar was maintained free of O_2 by a solution of sodium dithionite. Green growth required light, fructose, nitrogen gas and a limited amount of sulphide. A sulphide-stat was then constructed in which the N_2/CO_2 was first bubbled through a constantly refreshed sulphide solution so that the carry-over of sulphide into the culture medium maintained a constant sulphide concentration in the range of $5-10 \times 10^{-6}$ M. This facilitated detailed examination of nitrogenase activity and concomitant growth by the cyanobacterium.

Figure 1 shows some of the features of nitrogen-fixing growth by *P. boryanum*. After a lag of less than 1 day (some experiments had longer lags), growth proceeded logarithmically with a doubling time of about 24 h. On day 5 the culture began to lose colour, but logarithmic growth and full pigmentation were restored by the addition of fructose. The cyanobacterium would grow without a break in logarithmic growth on 0.2% fructose; Fig. 1 thus demonstrates a clear requirement for fructose. The specific activity of nitrogenase (as acetylene reduction) showed a transient peak after induction (similar to that seen following the induction of nitrogenase in *Gloeocapsa*³), reaching a specific activity of almost 5×10^{-8} M ethylene per min per mg protein, one of the highest reported for a growing cyanobacterium. Total

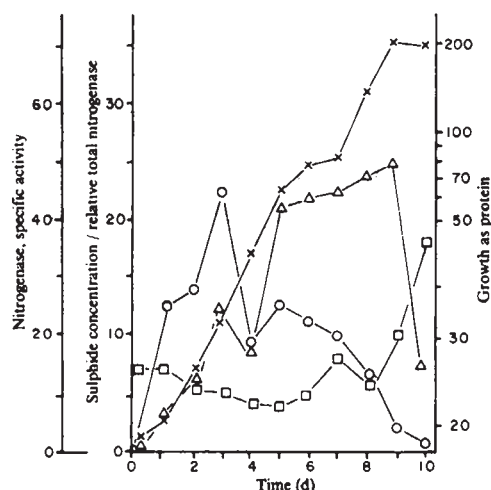


Fig. 1 Growth and nitrogen fixation by *P. boryanum* in the sulphide-stat. *P. boryanum* was grown under N_2/CO_2 in BG-11 (ref. 12) plus $NaNO_3$, collected and washed twice with BG-11 minus combined nitrogen, and then inoculated into the sulphide-stat. The growth vessel contained 400 ml BG-11 medium minus combined nitrogen plus 0.1% fructose buffered with $5/10^{-3}$ M N-Tris (hydroxymethyl) methyl-2-amino methane sulphonic acid, pH 7.8, and was maintained at $30^\circ C$ under 2,500-lux incident illumination. At a rate of 50 ml min^{-1} , 95% $N_2/5\%$ CO_2 was fed through a constant 500 ml of an anaerobic solution of 10^{-1} M-Tris (hydroxymethyl) amino methane, pH 7.4, 10^{-1} M sodium dithionite (which was refreshed at a rate of 4 ml h^{-1} by a similar solution containing 10^{-1} M Na_2S) and thence into the growth vessel. Strict anaerobiosis was maintained in the culture vessel and in nitrogenase determinations. Growth (x), scale units $10^{-6} \text{ g ml}^{-1}$, was monitored as protein by the Lowry *et al.*¹³ method as before¹⁴. Nitrogenase was assayed by the acetylene reduction technique¹⁵ as before¹⁴, except that the assay was performed in 10-ml plastic syringes (No. 7370, Pharmaseal Labs, Glendale), sealed with a piece of soft, translucent Teflon. Sodium dithionite (final concentration 2 mM) was added to each sample as a precaution against oxidation. Nitrogenase is expressed as relative total activity (Δ) (proportional to the ethylene evolved per min per ml), and as specific activity ($\circ$) (10^{-9} mol C_2H_4 per min per mg protein). Sulphide ($\square$), scale units 10^{-6} M, was measured using an Orion 94-161 electrode and antioxidant techniques described in Orion manual 94-161M (Orion, Inc., Cambridge, Massachusetts). The timescale (ordinate) is in days; parameters were measured at the same time each day. On day 5 de-aerated 10% fructose was injected to an additional 0.1% final concentration.

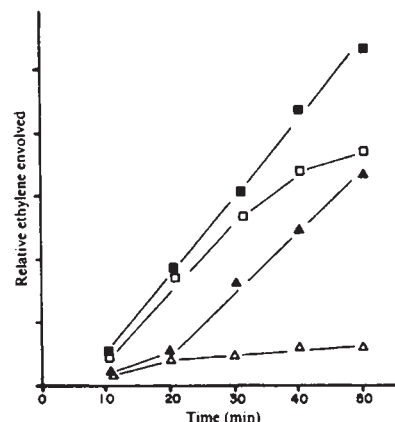


Fig. 2 Dithionite stimulation of nitrogenase activity (acetylene reduction) in cells grown in a sulphide-stat ($\blacksquare$, $\square$) and cells incubated without sulphide ($\blacktriangle$, $\triangle$). Open figures, no dithionite added to assays; closed figures, dithionite added (as in Fig. 1). Ordinate, time after addition of acetylene and dithionite. Abscissa, relative amount of ethylene evolved at the time indicated. Assayed as described in the legend to Fig. 1.

nitrogenase activity increased throughout the growth cycle. This is the first demonstration of concomitant logarithmic growth and nitrogen fixation by a blue-green bacterium lacking a cellular oxygen-protective mechanism. The sulphide concentration remained in the range of $5-8 \times 10^{-6}$ M until the end of the experiment when there was an increase. Except for the transient period between day 5 and day 7 the culture remained fully pigmented. A control culture without sulphide showed some growth, but with a much lower nitrogenase activity and with an almost complete lack of phycobiliproteins (as 625 nm absorbance). Figure 2 shows that the nitrogenase activity (acetylene reduction) in such a control culture was noticeably stimulated by added sodium dithionite, as demonstrated for similar cultures without fructose¹², whereas activity in a culture grown in a sulphide-stat was not. Thus, the sulphide-stat facilitates logarithmic, nitrogen-fixing, photoheterotrophic growth with complete pigmentation and sufficient metabolic energy to drive nitrogenase.

These observations suggest natural flexibility in the metabolism of *P. boryanum*. Presumably, carbohydrate replaces the carbon fixed through photosystem II-driven reactions and cyclic photophosphorylation generates ATP. The presence of most major pigments would suggest that photosystem II may be at least partially active.

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Low molecular weight circular and linear DNA in mitochondria from normal and male-sterile *Zea mays* cytoplasm

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Maternally inherited cytoplasmic variation in plants is well documented¹. One consequence of such variation in maize (*Zea mays* L.) is male sterility, sources of which have been classified into three groups (T, S and C) depending on nuclear gene fertility restoration²⁻⁴. These groups and the normal (N) male-fertile cytoplasm can be differentiated by restriction endonuclease analysis of mitochondrial DNA (mtDNA)^{5,6}, although differences have also been found within the N and C groups^{7,8}, and mitochondrial translation products^{9,10}. Encouraged by the finding that mitochondria of S-cytoplasmic types contain discrete small DNA species¹¹, we have looked for low molecular weight DNAs in mitochondria from plants with N, T, C and S cytoplasm. We report here that all four cytoplasm types contain supercoiled circular DNA molecules of approximately 1,940 base pairs. This is the first small supercoiled circular DNA to be found in higher plants. Its structure suggests it may be an autonomously replicated plasmid. N, C and S cytoplasm types also contain a DNA species of approximately 2,350 base pairs which is not present in T cytoplasm. C cytoplasm types contain two additional circular DNA species of 1,570 and 1,420 base pairs.

Figure 1 shows an electrophoretic analysis of mtDNA from the different cytoplasmic types. All bands are resistant to RNase but sensitive to DNase. There are three DNA bands which are common to all cytoplasm types. These are the supercoil (CCC), open-circle (OC) and linear (L) conformations of the same sequence (Fig. 1). Evidence for such a relationship between the three bands is: (1) S₁ nuclease treatment of CCC DNA produced OC and L molecules; (2) cRNA probes¹² of OC DNA hybridised to CCC, OC and L bands of mtDNA extracts on Southern blots¹³; and (3) electron microscopy¹⁴ showed the DNA from the CCC and OC bands to consist of circular, double-stranded molecules (Fig. 2a, b). The CCC conformations of the molecules were too small for positive identification in the electron micrographs but, during purification of the CCC DNA from the preparative gel and/or the spreading technique for electron microscopy, some molecules nicked to produce OC and L forms (46% were circular) (Fig. 3c, d). Similarly, some linear molecules were formed from DNA in the OC band (40% were circular) (Fig. 3a, b). The CCC and OC bands have a similar mean length of approximately 1,940 base pairs (assuming the Φ X174 standard to be 5,380 base pairs¹⁵) (Fig. 3a, c). We conclude that this common DNA species exists, in mitochondria, as a CCC conformation which nicks to OC and L forms during mtDNA isolation.

Mitochondrial DNA of T cytoplasm plants lack the 2,350-base pair DNA species found in N cytoplasm (labelled 'T' in Fig. 1). N and T lines from nine different nuclear backgrounds were compared and all produced the characteristic N or T pattern in Fig. 1. Mitochondrial DNA preparations from HA, RS and Q cytoplasm types also had the characteristic T pattern, indicating that these cytoplasm types are members of the T group^{3,4,10,16}. Six lines possessing T cytoplasm restored to male fertility by nuclear restoring genes produced the same pattern as male-sterile T types (Fig. 1, TRf track). Mitochondrial DNA was also obtained from two T seed sources which had been regenerated from callus tissue selected in the presence of *Helminthosporium maydis* race T toxin^{17,18}. The regenerated

plants were 100% male-fertile and fully resistant to the fungal toxin. However, their mtDNA produced the characteristic DNA banding pattern of T male-steriles.

S-group cytoplasm types possess two linear mtDNA species not found in other cytoplasm types (labelled 'S' in Fig. 1 and as described previously^{11,16,19}). S cytoplasm types restored to male fertility by nuclear restoring genes show the same pattern as S male-steriles^{11,19}.

C types possess two DNA species, not found in other cytoplasm types (labelled 'C' in Fig. 1). These DNA species were found in C types of the four different nuclear backgrounds examined, in RB cytoplasm which is a member of the C group^{3,4,10,16}, and in the one C cytoplasm line examined which had been restored to male fertility by nuclear restoring genes (Fig. 1, CRf track). Electron microscopic analysis¹⁴ of the C-specific DNA species showed the presence of double-stranded, circular molecules (Fig. 2c, d). The mean size of these circular molecules was about 1,570 base pairs for the slower migrating species (19% were circular) and about 1,420 base pairs for the faster migrating species (23% were circular) (Fig. 3e-h). Southern blot hybridisations¹³ showed that there is no sequence homology between the circular DNA species common to all cytoplasm types described above and the DNA species specific to S or C types.

Although our data do not demonstrate a direct relationship between the presence of these low molecular weight species and male sterility, several lines of evidence suggest a specific involvement of these DNA species in such sterility. Not only is the T-type cytoplasm the only male-sterile cytoplasm to lack a DNA band when compared to N but, significantly, plants carrying T cytoplasm exhibit the most extreme expression of male sterility of all the three male-sterile cytoplasm types. Plants carrying S and C cytoplasm types, however, possess additional mtDNA

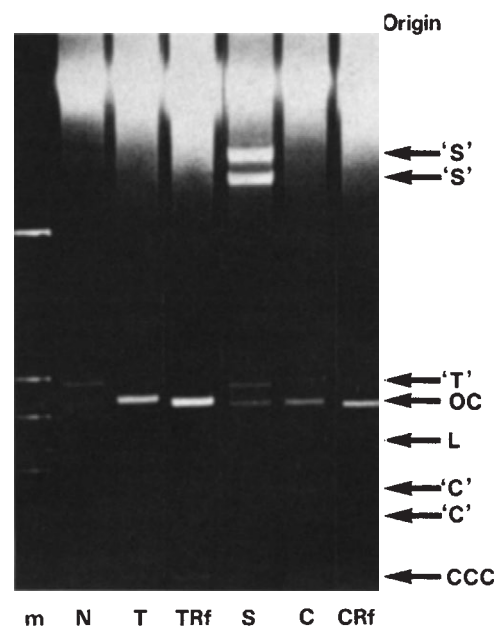


Fig. 1 Electrophoresis on 1.5% agarose gel of mtDNA preparations from N, T, TRf (T restored), S, C and CRf (C restored) cytoplasm types in B37 nuclear background. 'm' is a marker track of bacteriophage λ DNA digested with *Hae*III, the sizes of the bands are 3.9, 2.4, 2.1, 1.7 and 1.19 kilobases. 'S', 'T' and 'C' indicate band differences pertinent to S, T and C cytoplasm types respectively. CCC, OC and L indicate supercoil, open-circle, and linear bands, respectively. Etiolated shoots were grown, mitochondria purified, mtDNA isolated and electrophoresis carried out exactly as described elsewhere¹⁶. Cytoplasmic sources used were; N, T and TRf carried in A73, W59M, A495, W33, B37, B9A nuclear backgrounds. N and T were also used in CO192 $\times$ WJ, WF9 and F7 $\times$ F2. HA, RS and Q were in CO192 $\times$ WJ. C was in B37, WF9, A632 and CO192 $\times$ WJ. CRf was in B37. RB was in CO192 $\times$ WJ. S was in W13, W117, B37, WF9, CO192 $\times$ WJ. SRf was in B37. Eighteen members of the S group of cytoplasm types were used in CO192 $\times$ WJ.

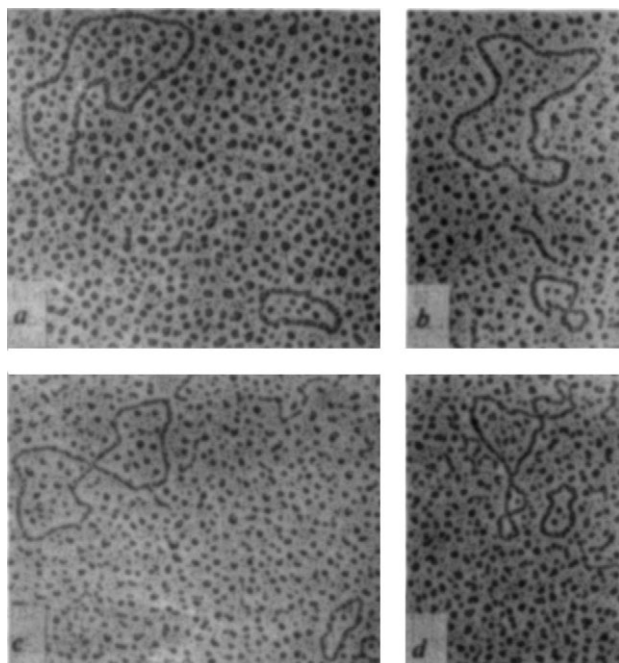


Fig. 2 Low molecular weight circular DNA molecules from mitochondrial preparations of C cytoplasm in CO192 × WJ nuclear background. DNA bands were isolated from a preparative agarose gel. *a*, OC band (see Fig. 1); *b*, CCC band; *c*, slower migrating 'C'-specific band; *d*, faster migrating 'C'-specific band. The large circular DNA molecule in each frame is ΦX174 bacteriophage included in each spread as an internal standard.

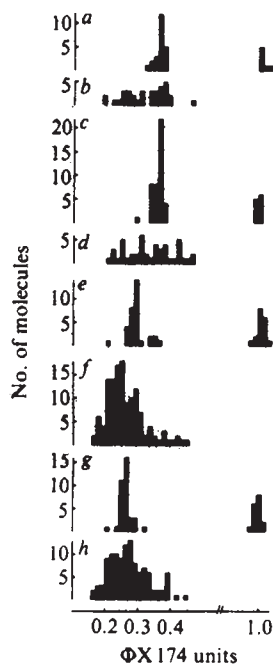


Fig. 3 Size-frequency distribution of DNA molecules from mitochondrial preparations as in Fig. 2. *a*, Circles from OC band (see Fig. 1); *b*, linear from OC band; *c*, circles from CCC band; *d*, linear from CCC band; *e*, circles from the slower migrating 'C'-specific band; *f*, linear from the slower migrating 'C'-specific band; *g*, circles from the faster migrating 'C'-specific band; *h*, linear from the faster migrating 'C'-specific band. The size distribution of the ΦX174 molecules used as internal standards with each spread is also shown. Photographic negatives of the electron micrographs were enlarged and the length of the DNA molecules measured with a map measure.

species not found in each other or N and T types and frequently exhibit only incomplete male sterility. In fact, some S types have reverted to complete fertility in the absence of any known nuclear restoring genes²⁰.

This finding of six different kinds of low molecular weight DNA molecules in maize raises many interesting questions in addition to their role in pollen fertility. It is clearly important to investigate their origin and their mode of replication. We are now studying their sequence relationship to other DNAs of the cell. If they are self-replicating they are potentially very useful tools for the study of plant transformation.

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A plasmid associated with virulence in the marine fish pathogen *Vibrio anguillarum* specifies an iron-sequestering system

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Many of the high-virulence strains of the marine fish pathogen *Vibrio anguillarum* isolated from epizootics of the widespread fish disease vibriosis, harbour a specific plasmid class which is absent from low-virulence strains¹. Curing experiments have confirmed a link between this specific plasmic class and the ability of *V. anguillarum* to establish infections². In general, all bacterial virulence factors promote growth in the antagonistic environment of the host defence mechanisms. One line of defence is provided by the proteins transferrin and lactoferrin³⁻⁵, which bind iron, rendering it unavailable to pathogens. A mechanism whereby invading bacteria may successfully compete for the otherwise unavailable iron could therefore become crucial in enabling them to proliferate in body fluids and tissues. I report here evidence which shows that the *V. anguillarum* virulence plasmid specifies a very efficient iron-sequestering system enabling bacteria to survive in conditions of limited iron availability.

To determine whether a plasmid-mediated iron sequestering mechanism exists in *V. anguillarum*, the growth kinetics of selected *V. anguillarum* biotype I⁶ strains of high virulence and their cured low virulence derivatives were studied in minimal

Table 1 Correlation between presence of plasmid, ability to grow in presence of transferrin and virulence of strains of *Vibrio anguillarum*

<i>V. anguillarum</i> strain	Presence of plasmid	MM	Generation time (h) in MM + transferrin	MM + transferrin + iron	Virulence (LD ₅₀)
775(pJM1)	pJM1	1.0	1.1	1.1	1.0×10^3
775 (pJM11)	pJM11	1.0	1.2	1.0	3×10^3
LS173(pJM1)	pJM1	0.9	1	1.0	2×10^3
133S(pJM1)	pJM1	1.1	0.8	0.9	1.3×10^3
H-775-850	pJM11	1.0	0.9	1.1	0.9×10^3
E-775-300	pJM11	1.1	1.0	1.0	1.5×10^3
H-775-1	No plasmid	1.1	4.0	1.2	2.1×10^7
E-775-100	No plasmid	1.0	3.5	1.0	2×10^6
286D	No plasmid	1	2.7	1.0	1×10^8
NCMB1291	No plasmid	0.8	2.9	1.1	2×10^7

Clones were analysed for plasmid DNA by agarose gel electrophoresis as before⁸. pJM11 was generated by transposition of the TnA sequence containing the ampicillin resistance genes to the original *V. anguillarum* virulence plasmid pJM1 (ref. 2). Growth curves in minimal medium (MM), MM + transferrin (2.3 μ M) or MM + transferrin (2.3 μ M) + iron (0.2 mM FeCl₃) were obtained as described in Fig. 1. Generation times were calculated on the portion of growth curves between 6 and 14 h of growth at 22 °C. Virulence was tested on juvenile coho salmon (*Oncorhynchus kisutch*) weighing about 14 g as before¹. Virulence is quantified as LD₅₀ values (number of microorganisms that will kill 50% of the animals inoculated) as determined by the Reed-Muench method⁹.

medium in the presence of transferrin. This protein binds the trace amounts of iron present as an impurity (about 3 μ M) in minimal medium, making it unavailable for bacteria growth. The strains used were the plasmid-containing, high virulence 775 (pJM11) and its isogenic low virulence derivative H-775-3 obtained by curing the pJM11 plasmid from 775 (pJM11) at 37 °C, a temperature higher than its optimal growth temperature².

Figure 1a shows that addition of transferrin (final concentration 2.3 μ M) to the culture medium inhibited the growth of the low virulence plasmid-less H-775-3 strain. Addition of excess iron (as 0.2 mM FeCl₃) to the medium totally reversed the inhibitory effect of transferrin by saturation of the iron-binding sites on the protein molecule. Similar results were obtained with other cured derivatives obtained by either exposure to 37 °C (H-775-1) or to ethidium bromide (E-775-100) (Table 1). Figure 1b shows the growth kinetics for the high virulence parent strain 775 (pJM11) containing the virulence plasmid. Growth of this strain is not affected by the concentration of transferrin which is inhibitory for the cured strains. Table 1 shows that those clones which were exposed to 37 °C (H-775-850) or to ethidium bromide (E-775-300) during the curing treatments but retained the plasmid still had a high virulence phenotype. It also shows that they could grow in the presence of transferrin, indicating that the curing procedures had not modified the pathogenic properties or ability to utilise complexed iron of bacteria other than in those cured of their pJM11 plasmid. Analysis of other high and low virulence strains isolated from different locations¹ showed similar correlations between segregation of pJM11, attenuation of virulence and inability to grow in the medium with transferrin (Table 1). My results thus suggest that the *V. anguillarum* virulence plasmid specifies a very powerful iron sequestering system which enables *V. anguillarum* to utilise highly complexed iron *in vitro*.

Is the possession of this plasmid-mediated iron uptake system a selective advantage *in vivo* for *V. anguillarum* infections? The results in Table 2 strongly support this contention. In experimental infections of fish with low-virulence cured derivatives of *V. anguillarum*, the mean lethal dose values (LD₅₀) are decreased about 300-fold if iron is included in the inocula, while added iron does not affect the LD₅₀ value in infection with the plasmid-containing strain 775 (pJM11). Thus there is a greater selective advantage for the plasmid-containing strain only when iron is not included in the inoculum. These results, of course, do not preclude the existence of other chromosomal or plasmid-mediated virulence determinants, which can play a part in other stages of the invasion process. However, considering the results reported here, it is possible to speculate that breeding and selection for transferrin genotypes of salmonids which are more resistant to the *Vibrio anguillarum* plasmid-mediated iron

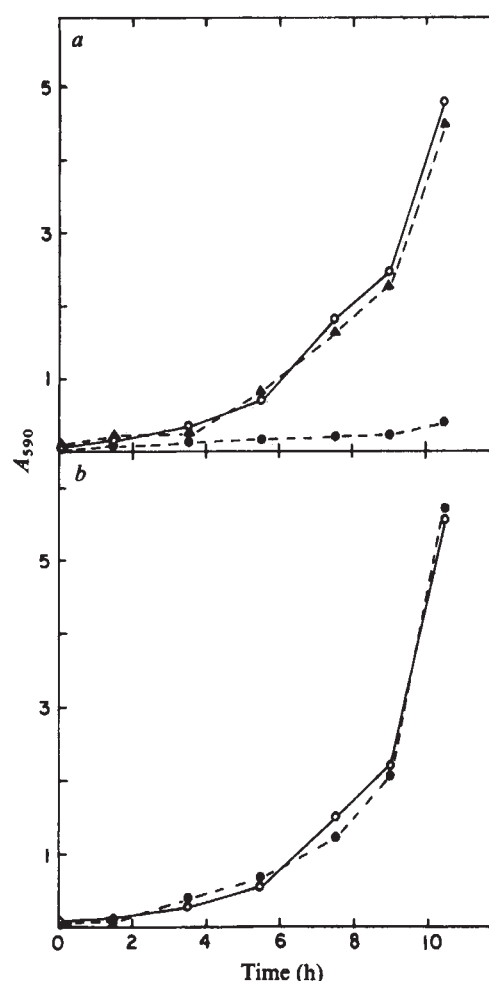


Fig. 1 Effect of transferrin on the growth rate of low virulence (a) and high virulence (b) *V. anguillarum*. Growth curves were obtained for: ○, minimal medium; ●, minimal medium plus 2.3 μ M transferrin (Sigma); ▲, minimal medium plus 2.3 μ M transferrin and 0.2 mM FeCl₃. Bacteria were grown overnight at 22 °C in a glucose-casamino acids (Difco) M9 salts minimal medium. Those cultures were used to inoculate sets of 250 ml flasks containing 100 ml of fresh similar medium at 22 °C with the different additions. Absorbance at 590 nm (A_{590}) was measured using a Gilford-vacuum micro-sample spectrophotometer. Samples were withdrawn every 2 h and the A_{590} was determined. a, Low virulence H-775-3 strain obtained by curing plasmid pJM11 (Ap^r) from 775 (pJM11); b, high virulence *V. anguillarum* 775 (pJM11).

Table 2 Effect of iron on experimental infections of fish with cured low-virulence strains of *V. anguillarum*

Strain	LD ₅₀ control	LD ₅₀ with iron	Increase in virulence
H-775-3	2.1 × 10 ⁷	8.2 × 10 ⁴	256-fold
H-775-7	3.2 × 10 ⁶	8.7 × 10 ³	368-fold
E-775-100	2.1 × 10 ⁶	9.0 × 10 ³	233-fold
775 (pJM11)	4.3 × 10 ³	3.3 × 10 ³	1.3-fold

Control LD₅₀ values were determined as described in Table 1. LD₅₀ values with iron were determined by including 86 µg iron (as ferric ammonium citrate) in the bacterial inoculum. In all cases, inocula were injected subcutaneously (0.1 ml) behind the fish dorsal fin. No controls, into which 86 µg iron (as ferric ammonium citrate) in saline solution was injected, died.

sequestering system could open new avenues to the control of fish disease. Differences in susceptibility of salmonids with different transferrin genotypes have previously been shown in infections with the causative agent of bacterial kidney disease⁷. Experiments on this trend as well as the characterisation of the iron sequestering system in *Vibrio anguillarum* are in progress.

Williams has described¹⁰ another plasmid-mediated iron-uptake system as being specified by the Col V plasmid of those invasive strains of *Escherichia coli* associated with cases of bacteraemia in man and domestic animals. It would be interesting, both from an evolutionary as well as an epidemiological standpoint to assess whether the *V. anguillarum* and *E. coli* plasmid-mediated iron uptake systems are related.

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Study of haem structure of photo-deligated haemoglobin by picosecond resonance Raman spectra

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It is well known that the oxygen affinity of haemoglobin depends on the number of combined oxygen molecules¹. This cooperative effect is considered to arise from a reversible protein transition between two forms which differ in tertiary and quaternary structure². However, the various steps of the structural changes concerning the protein and the haem have not been identified. Using time-resolved spectroscopy coupled to flash photolysis, we have attempted to elucidate the influence of protein on the relaxation processes of haem in haemoglobin. We now report our first results obtained in a picosecond time-resolved resonance Raman study of haemoglobin.

Resonance Raman scattering from haemoglobin solution was induced by light pulse at 532 nm obtained by second harmonic generation from a YAG modelocked Quantel laser³. Two different temporal pulse lengths—15 × 10⁻⁹ and 30 × 10⁻¹² full width at half maximum (FWHM)—were used; the maximum energy delivered per pulse was 300 mJ and 40 mJ, respectively. By means of a cylindrical lens focusing⁴ (focal length = 750 mm), the laser beam was passed through an aperture (0.2 mm) to the haemoglobin sample. The cell (10 × 20 mm) containing the sample was put into another cuvette (30 × 30 mm) containing the same buffer as the sample. The laser beam arrived at normal incidence on one side of the first cell and approximately 30° to one side of the sample cell. This arrangement gives a good scattered signal at 90° from the incident beam, permitting the use of lower laser power and consequently, reducing the reflection. The residual Rayleigh scattering of the sample was reduced by placing an interference filter in front of the entrance slit of a polychromator (used in the first order) with an 1,800 grooves mm⁻¹ holographic grating. The detection system was similar to that used by Bridoux and Delhay⁵. The photocathode of a front stage image intensifier tube (EMI 9912) was located in the focal plane of the spectrograph. This system can give a light gain of 10⁶ at the maximum photocathode sensitivity. We have chosen a gain of 10⁵. The picture on the target outlet of the intensifier tube was taken up by a vidicon SEC camera (EMR). The spatial resolution of the system was 5 lines mm⁻¹, with a sensitivity equivalent to ASA × 10⁸. The spectral information was obtained in a multi-channel analyser system (Didac 800) and recorded in an X-Y plotter. In all cases, to eliminate back-lash effects in the grating, the spectrometer was calibrated using the 586.0, 583.4, 580.3 and 573.9 nm lines of an argon lamp. For the recording of the spectra the input slit was set at 0.2 mm, corresponding to a theoretical spectral resolution (FWHM) of 4 cm⁻¹. The technical arrangement is shown in Fig. 1.

Haemolysates were extracted from fresh human adult blood, and membrane-free samples were obtained by centrifugation at 18,000 r.p.m. (ref. 6). Haemoglobin A was stripped of ions on a mixed-bed ion-exchange resin AG501 × 8 (Biorad)⁷. The oxygenated haemoglobin solution was buffered in 0.1 M potassium phosphate at pH 7. Deoxyhaemoglobin (Hb) was obtained by addition of a small fraction of sodium dithionite into buffered oxygenated haemoglobin (HbO₂). At room temperature Hb was converted to the carbon monoxide complex (HbCO) by using CO gas at a pressure of 1 atm above the solution. During the experiments the temperature was held at 22 °C. All solutions for the laser runs were at a concentration of 3 × 10⁻³ M calculated on the basis of haem. As the optical density of the sample is very high ($A_{1\text{cm},532} = 35$), the weak peak intensity (6 GW cm⁻²) used from the picosecond flash laser cannot initiate stimulated processes in the haemoglobin solution. Nevertheless, in the same optical geometric conditions, picosecond spectra show a background noise higher than that of nanosecond spectra. This noise may be due to energy dissipation processes of the electronically excited haem states⁸ produced by the high-power density laser of the picosecond pulse.

Nanosecond time-resolved resonance Raman spectra represent an average result of several laser shots (15–20 laser pulses). However, each picosecond time-resolved resonance Raman spectrum was obtained with the accumulation of 100 laser pulses (at least 2 s between each pulse). In these conditions, artefacts with repeated picosecond laser pulses are avoided because haemoglobin is always completely religanded in less than 1 s. Not all spectra were smoothed out or normalised for intensity.

A typical transient picosecond resonance Raman spectrum of the haem structure-sensitive spectral region (1,300–1,700 cm⁻¹) of haemoglobin is shown in Fig. 2b. Both direct haem-protein interaction, which should change the porphyrin backbone symmetry, and iron electronic states variations, alter most of the stretching frequencies of C–C and C–N bonds in the porphyrin ring which are seen in the Raman spectrum range between 1,300

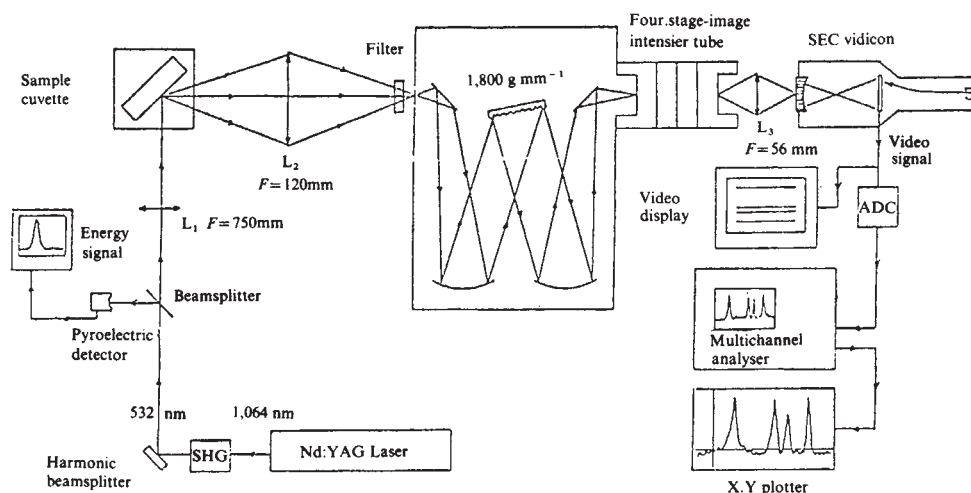


Fig. 1 Experimental arrangement.

and $1,700\text{ cm}^{-1}$ (refs 9–12).) This spectrum seems to be the superposition of spectra of HbO_2 and stable deligated haemoglobin (Hb) (Fig. 2) and reflects the general pattern observed with nanosecond Raman spectra.

Relative amplitude changes between spectral bands have not been studied and correlated with laser energy. However, no laser pulse intensity dependence has been detected in the frequency positions of the transient picosecond haemoglobin spectra with an excitation wavelength of 532 nm, the frequencies of the observed bands were $1,586 \pm 2$ and $1,640 \pm 2\text{ cm}^{-1}$ for oxyhaemoglobin and $1,554 \pm 2\text{ cm}^{-1}$ for deoxyhaemoglobin. This result suggests that a Hb-like photoproduct was generated from HbO_2 , and that this photoproduct appeared in 30 ps.

The oxygen photo-deligation process is complete within 2.5 ps (ref. 13) and is initiated with the protein in the oxygenated structure (*R* configuration). A recent study of the photodissociation of HbCO by time-resolved absorption spectroscopy indicates that the iron-ligand linkage break leads to a readjustment of the haem in its protein pocket. This haem-protein readjustment occurs in microseconds¹⁴. The evolution to a more stable quaternary *T* state seems to take longer¹⁵. Also, changes in porphyrin structure caused by deligation and detected by picosecond time-resolved resonance Raman spectra cannot be associated with the protein constraints exerted on the haem; these include the imidazole strain and Van der Waals contacts. Thus, the recent finding that only a very short iron motion exists between oxy- and deoxyhaemoglobin¹⁶ confirms that the differences between the resonance Raman spectra of these compounds depend primarily on the iron coordination states (liganded or not).

From these results, we conclude the following. (1) The transient picosecond resonance Raman spectrum of HbO_2 is essentially due to a reorganisation of the porphyrin core through a change in the electron distribution resulting from the departure of the oxygen ligand. (2) The time required for the reorganisation in the structure of the porphyrin core is less than 30 ps. (3) The picosecond time-resolved resonance Raman spectra of haemoglobin are related to the haem structural state with the iron atom pentacoordinated in a *R* protein conformation before its return to the *T* stable position. (4) The picosecond time-resolved Raman data show that the haem structure is at most only slightly dependent on the protein conformations, and that the porphyrin backbone structure is not very flexible. This conclusion agrees with X-ray experiments¹⁷.

Up to now, using CARS¹⁸ and the nanosecond time-resolved resonance Raman technique^{19–21}, no significant transient constraints have been observed between photodissociated HbCO and stable deoxyhaemoglobin. Because the change in haem geometry is not associated with the *R* or *T* globin state, it is conceivable, however, that the non-equilibrium globin structure brings about some reorientation between the haem and the globin and slight changes in the haem electronic repartition.

These relaxation processes must be more sensitive to electronic absorption spectra than to resonance Raman spectra. Lyons *et al.* could have seen this effect in the slight spectral shift of 3 cm^{-1} between unliganded haem from stable deoxy- and photodissociated haemoglobin²¹. Unfortunately, the digital technique with our dispersion is not refined enough to obtain a precision of better than 4 cm^{-1} on the absolute frequencies, and in the present work, we cannot draw clear conclusions on the existence of this small shift.

Using conventional continuous-wave resonance Raman spectroscopy, previous workers have already observed that the porphyrin spectra are not modified by the *R* and *T* shapes of apoprotein^{22,23} and that the haem structure (liganded or not) is independent of the apoprotein nature—amino acid composition, protomere associations, chemical modification²⁴. Nevertheless, with respect to normal haemoglobin, only very slight shifts in Raman frequencies have been reported for a modified haemoglobin²⁵. As the oxygen affinity of haemoglobin depends

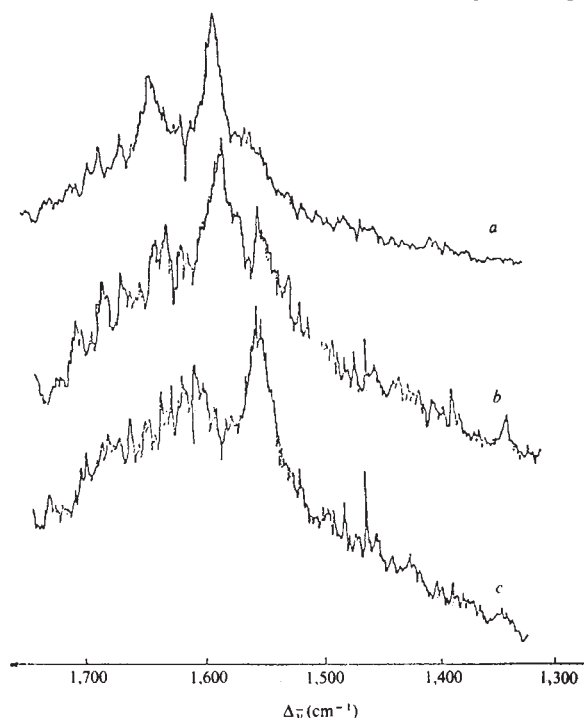


Fig. 2 Transient resonance Raman spectra. *a*, Fully oxygenated haemoglobin; excitation 15 ns, 15 laser pulses, 3 mJ per pulse. *b*, Partially photo-deligated haemoglobin; excitation 30 ps, 100 laser pulses, 1.2 mJ per pulse. *c*, Unliganded haemoglobin from stable deoxyhaemoglobin; excitation 15 ns, 20 laser pulses, 3 mJ per pulse. The same spectra were obtained from photo-deligated HbCO .

on the integrity of the protein structure^{26,27}, we suggest that the protein plays its part by acting directly on the charge of the iron atom and does not affect the geometric state of the haem. Furthermore, the protein relaxation process should be influenced by the structural and electronic state of the haem (including the iron ionic state, iron bound or not). This may explain some ambiguities and disagreements between recent electron paramagnetic resonance measurements²⁸, EXAFS data¹⁶ and resonance Raman work^{24,28,29}.

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Transient Raman study of CO-haemoprotein photolysis: origin of the quantum yield

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Carbon monoxide and haemoproteins (Fe²⁺) form ligand-protein complexes that are photodissociated with high quantum efficiency by visible light^{1,2}. By photolysing the CO-protein complex with pulsed laser radiation it is possible to generate transient species with properties which reflect both the nature of the photolytic and recombination processes as well as non-equilibrium protein dynamics. Using a single pulse both to photolyse and examine the sample, we previously reported³ the resonance Raman spectrum of a transient species of haemoglobin (Hb) occurring within 10 ns of the photodissociation of carboxyhaemoglobin (HbCO). Transient species generated by photodissociating HbCO have been intensively studied by using transient absorption spectroscopy covering picosecond^{4–6}, nanosecond^{7–10} and longer time scales^{11–15}. The overlap of the absorption bands related to various species can hamper the extraction of quantitative information from absorption spectra. Nevertheless, progress has been reported, in particular, the results of nanosecond transient absorption studies^{9,10} reported after the experiments described here were well under way. Those results, including re-interpretation of earlier data⁸, led to the suggestion^{9,10} that photolysis of HbCO actually leads to a near-unity quantum yield of a deoxy-Hb species, followed by substantial recombination within 100 ns. We present here definitive evidence from Raman spectra of the 1,350–1,380-cm⁻¹ region, where the HbCO and deoxy-Hb species exhibit well separated peaks, that this interpretation is indeed correct. We have also measured accurately the HbCO population as a function of delay time after full photolysis, and thereby measured the kinetics of the recombination process. Our findings bear directly on such questions as geminate recombination and the origin of the difference in quantum yield of photodissociation for HbCO and carboxymyoglobin (MbCO). These two systems exhibit very different quantum yields when strongly pumped in the visible: MbCO undergoes 97% photolysis whereas HbCO undergoes only 45–47% (ref. 15).

The apparatus used was similar to that reported previously³, except that the resonance Raman spectrum was generated by a 10-ns dye laser pulse (Moletron UV 24 and DL 14) fired with

an adjustable delay (up to 1 ms) after the pump pulse from the frequency doubled (5,320 Å, 20 mJ) output of a ND:YAG laser (Holobeam 500 QG). The spectra discussed below were obtained using between 4,100 Å and 4,350 Å (≤0.1 mJ) probe pulse excitations. As before³ the spectrum is accumulated by an optical multichannel analyser, based on an SEC Vidicon tube. The sample configuration has been changed to a flowing cell with the temperature and atmosphere of the sample under external control. A more detailed account of the apparatus is reported elsewhere¹⁶. Both Hb and Mb solutions (0.1 mM in haem, pH 7.5 phosphate buffer) were maintained at 10–20 °C under one atmosphere of CO. The solutions were initially degassed and treated with dithionite.

Spectra were recorded covering the region 1,225–1,575 cm⁻¹. The most prominent feature in this spectral region for Soret band excitations is the polarised peak which occurs at 1,357 cm⁻¹ for deoxy-Hb, 1,373 cm⁻¹ for HbCO and at 1,377 cm⁻¹ for HbO₂ with similar values for Mb. The present study will deal only with the behaviour of this peak. A series of typical spectra for Hb and Mb is shown in Figs 1 and 2, respectively. Firing the dye laser before the photolytic pulse (a), we find that the spectrum reflects predominantly unphotolysed material (photodissociation by the probe pulse has been minimised by focusing the beam to a line). Within the first few nanoseconds after photolysis, the HbCO and MbCO peaks have completely disappeared and a peak in the region of the deoxy species has appeared (b). This observation is consistent with previous results obtained using a single pulse^{3,17,18} and using CARS¹⁹. At later times a difference is observed in the two systems. In the Hb system the HbCO peak regains nearly 50% of its intensity (Fig. 1c) within 100 ns of photolysis. On the same time scale (Fig. 2c) no corresponding change is observed in the Mb spectrum. At much later times the expected bimolecular recombination¹³ takes place in both Hb (Fig. 1d–g) and Mb (Fig. 2d–f). The transient deoxy peak is of better quality in the spectra excited with 4,200 Å and its position at early times (<1 μs) is shifted slightly to lower energy than that associated with the steady-state deoxy-Hb (T) species. This shift is probably due to the effect of the structure of the haem pocket on the porphyrin^{20,22}.

At first glance, the intensity of the deoxy peak in the Hb system (Fig. 1) seems to be constant during the fast recombination process. This suggests that a species may exist after photolysis which is 'silent' in Raman scattering, and which subsequently decays into HbCO. To analyse this possibility more carefully, we used a nonlinear least-squares analysis to fit spectra similar to Fig. 1c–g, at 10 ± 2 °C, to a weighted average of

spectra similar to Fig. 1a, b.

$$S(\Delta\nu) = A_{\text{CO}}S_a(\Delta\nu) + A_{\text{deox}}S_b(\Delta\nu) + B$$

where $S_{a,b}(\Delta\nu)$ represent the corresponding points of the spectra in Fig. 1a, b. The three parameters A_{CO} , A_{deox} and B are varied to achieve a least-squares fit to $S(\Delta\nu)$ over the range shown. The results of this analysis are shown in Fig. 3, where we plot the weighting factors A_{CO} and A_{deox} against delay time. It is significant that, although all three parameters were varied independently, the value of B was consistently insignificant (<5% of the peak) and the sum of A_{CO} and A_{deox} was consistently between 95% and 110%. These results were so striking that we decided to reject the two points (out of 28) for which they did not apply. The quality of the fit was quite good for times below 400 ns. Data were taken at excitation frequencies from 4,350 Å, where the ratio of the deoxy-Hb Raman cross-section to that for HbCO is about 3.0, to 4,150 Å, where it is about 0.3. As shown in Fig. 3, the population values determined by our analysis, in the range studied, did not depend on excitation frequency outside an experimental error of about 10% for delays below 400 ns. Thus, we conclude that (1) for times between 10 and 400 ns, the Raman enhancement does not change appreciably for either species, nor do their respective line shapes; (2) for each ligated haem which appears, an unligated haem disappears; (3) 41% of the photolysed haem groups initially observed at 15 ns after photolysis recombine within 200 ns. A fit to the points under these assumptions, shown in Fig. 3, gives a recombination lifetime of 65 ns for the fast process. The occurrence of such a fast recombination process has also been inferred^{9,10} from transient absorption studies. Note that the absorption lines of the two species are not completely resolved spectrally.

The analysis discussed above was only qualitatively useful beyond 400 ns, because the position of the peak for the deoxy-Hb species begins to shift noticeably at that delay. The deoxy peak moves 3–4 cm^{-1} , to higher energy, between 0.5 and 4 μs delay. The resonant enhancement also evidently increases. (The amount of this increase is a function of excitation frequency, as expected. At 4,200 Å the increase is about 10%). These changes, probably the spectral signature of the initial events

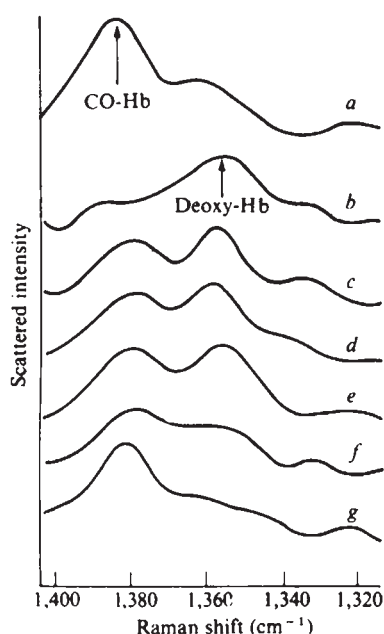


Fig. 1 Raman spectra of HbCO taken at various delays after photolysis with a time resolution of 10 ns. The various traces, shown displaced vertically for clarity, correspond to: a, before photolysis; b, 15 ns after; c, 100 ns; d, 1 μs ; e, 10 μs ; f, 100 μs ; g, 1 ms. The small shifted 'CO' peak in trace b may be due to the presence of a small amount of HbO₂. Note the reappearance of HbCO in trace c.

heralding the R–T transition in the partially ligated Hb²², caused spurious changes in the population values extracted in the least-squares analysis, and the resulting fits were noticeably poorer. Hence, the results for these later times could not be treated as quantitatively as those displayed in Fig. 3. Nevertheless, the results at early times yield striking evidence that the quantum yield for photolysis of HbCO deviates from 100% due to a recombination of photolysed haems, with a characteristic time of 65 ± 25 ns. Correcting our results for the recombination taking place in the first 15 ns, we obtain an estimated quantum yield of $54 \pm 4\%$ (that is, 46% recombination total). Comparing this with the reported value of the quantum yield of 47% (53% unphotolysed) measured at longer times, we see that the absolute quantum yield for the primary photolysis process (that is, the Fe–CO bond cleavage) is about 90%, a value similar to the total yield observed in MbCO, where the fast recombination does not occur.

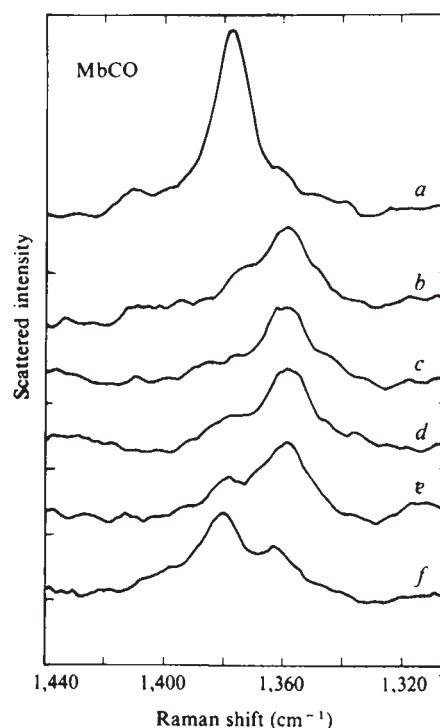


Fig. 2 Transient Raman spectra of MbCO analogous to Fig. 1. The traces represent: a, before photolysis; b, 15 ns after; c, 100 ns; d, 1 μs ; e, 10 μs ; f, 1 ms. No significant recombination is observed for times <100 μs .

The observation of the nanosecond recombination in Hb but not in Mb and the known similarity in electronic properties of the haems in both proteins tend to rule out explanations for the occurrence of this sizeable fast recombination based solely on the electronic properties of the iron porphyrin. On the other hand, there are germane properties of haemoproteins, such as the quantum yield for the photodissociation of the CO–haem complex, which are dependent not only on the specific protein but also on the quaternary structure of a given haem protein¹⁵. Consequently, it seems highly plausible that variation in protein structure about the haem rather than major variations in the electronic state determinants of the photolytic pathway is responsible for the major differences²¹ in the behaviour of photolysed carboxyhaemoproteins.

The rapidity of this process suggests that the recombination process involves photodissociated CO molecules that have remained in the vicinity of the haems, that is, geminate recombination. This interpretation is strongly supported by our observation that the fast recombination is insensitive to the CO concentration (0.02–2 atm). This lack of sensitivity to the CO

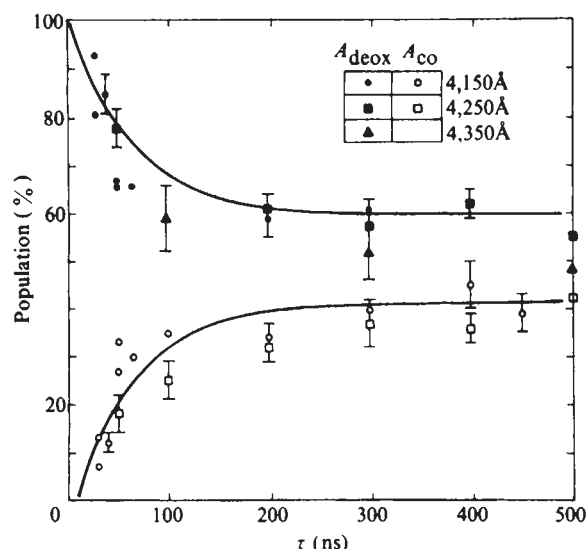


Fig. 3 Population weight factors, A_{deox} , A_{CO} , resulting from the least-squares analysis described in the text. The wavelengths in the key denote the dye laser excitation used to obtain the resonance Raman spectra.

concentration was also observed in transient absorption measurements at discrete frequencies¹⁰. In the absorption study it was also found⁹ that the amount of recombination decreased with increasing temperatures. From these dependencies we conclude that the fast recombination is, in fact, geminate in origin.

The deoxy-haem species appears in transient absorption spectra within picoseconds of photolysis⁴⁻⁶. Hence, any geminate recombination mechanism must include some means of trapping the CO molecule in the haem pocket after cleavage of the Fe-CO bond for a time τ of about 65 ns. Furthermore, this trapping, if it involves the Fe ion, must occur in a way that does not produce the HbCO absorption or Raman spectra. It seems likely, then, that the trapping is related to direct interaction of the CO molecule with the surrounding protein. This interaction probably takes the form of CO-binding sites (or potential minima) on the protein. This direct CO-protein interaction, responsible indirectly for the geminate recombination, would explain the protein and conformation dependence of the quantum yield.

The difference between the MbCO and HbCO systems could lie either in the dynamics of escape and recombination from similar sites, or in the existence of different kinds of sites. In either case, the α - and β -chains would be expected to exhibit differences in their behaviour. The conformation dependence of the quantum yield¹⁵ may also be explained on this basis. Furthermore, the proximity of the geminate recombination fraction to 50% in the *R* conformation strongly suggests that one chain predominantly undergoes recombination and the other does not. Indeed, experiments with isolated chains do indicate differences¹² at 290 K ($QY_{\beta} \approx 8\%$, $QY_{\alpha} \approx 90\%$). However, because this relationship is strongly temperature dependent, it may not hold at physiological temperatures, where the quantum yield for HbCO is much higher, and the geminate recombination is correspondingly reduced.

Thus, we have shown that transient resonant Raman spectra of photodissociated HbCO support the basic features of the suggestions made recently on the basis of transient absorption experiments^{9,10}. The better separation of the transient species in the Raman spectra has made possible a more complete determination of the populations as a function of time after photolysis. Our findings indicate that differences in the quantum yield for photodissociation of carboxyhaemoproteins can originate from differences in geminate recombination rates.

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Correlation of IR spectroscopic, heat capacity, diamagnetic susceptibility and enzymatic measurements on lysozyme powder

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The interaction between protein and water is of fundamental importance for processes ranging from protein folding and enzymatic activity¹ to anhydrobiosis². In this letter we bring together results from diverse types of measurements to give a unified picture of the hydration process for lysozyme. The data come principally from experiments with protein films and powders. The principal aim is to examine the relationship between the sites of water interaction, the extent of coverage, and the enzymatic activity, thus providing a better understanding of the relationship between water and enzyme dynamics³.

Figure 1 describes the results of measurements on the heat capacity⁴, enzymatic activity⁵, IR spectroscopic properties⁶, and diamagnetic susceptibility⁷ of lysozyme powders as a function of hydration level (h , g of water per g of protein).

The appearance of a positive peak at $1,580\text{ cm}^{-1}$ in the IR difference spectrum (Fig. 1a) indicates the formation of carboxylate species⁸. Apparently, drying the protein below $0.05h$ has produced inversion of the pK order for carboxylic and basic groups, resulting in charge neutralisation by proton transfer. This implies that interaction with water is associated with proton redistribution—that is, normalisation of the pK order with deprotonation of carboxylic acid groups and protonation of basic groups, presumably amino groups, to give the ionisation state found for the protein in solution. A proton redistribution process is expected to produce a rise and fall in the heat capacity, as is observed at $0.05h$ (Fig. 1d).

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The discontinuity in several properties at 0.07h must represent at least changes in water–water and water–protein interactions, because there are effects on the IR spectroscopic properties of both water (Fig. 1c) and protein (Fig. 1a, b). The change in slope of the heat capacity function at 0.07h suggests that the water–protein arrangements have greater freedom above the discontinuity. In this regard, the partial specific heat capacity of the water bound below 0.07h is similar to that of ice or water vapour, while that above 0.07h is greater than that of liquid water, which has twice the specific heat capacity of ice. It is important that the fine structure of the amide I' band is unchanged above 0.07h, and thus significant contributions to the heat capacity from changes in protein conformation can be ruled out⁶.

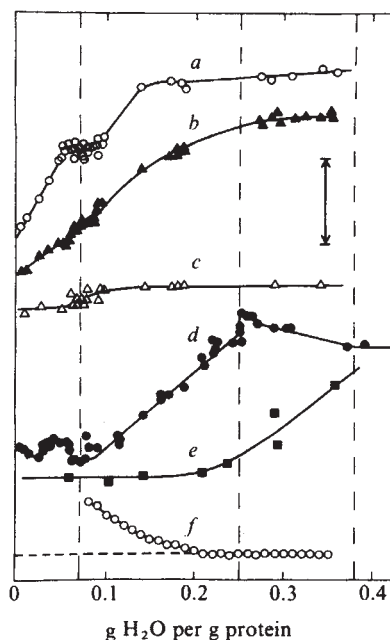


Fig. 1 Properties of the lysozyme-water system as a function of water content. The correspondence between the vertical bar in the figure and the units of measurement is given separately for each curve. From top to bottom the curves are: *a*, Absorbance at the carboxylate band maximum ($1,580\text{ cm}^{-1}$), measured at 38°C ; units, 0.35 A . *b*, Change in the amide I' band, measured at 38°C as the difference between the negative ($1,690\text{ cm}^{-1}$) and positive ($1,645\text{ cm}^{-1}$) extrema about the isosbestic point of the differential spectrum; units, 0.17 A . *c*, Frequency of the highest intensity maximum of the OD stretching band of adsorbed D_2O , measured at 38°C . The total shift is from $2,550\text{ cm}^{-1}$ to $2,580\text{ cm}^{-1}$. *d*, Apparent specific heat capacity of lysozyme, for 25°C ; units, $0.2\text{ J K}^{-1}\text{ g}^{-1}$. This function is a measure of the excess heat capacity of the system per g protein. *e*, Enzymatic activity, measured at 25°C ; units, $5 \times 10^{-6}\text{ s}^{-1}$. *f*, Diamagnetic susceptibility, measured at 25°C and expressed as differential susceptibility per g of water adsorbed; units, $0.55 \times 10^{-6}\text{ e.m.u. g}^{-1}$. The high hydration limit is equal to the value for liquid water ($0.721 \times 10^{-6}\text{ e.m.u. g}^{-1}$).

The continuous changes in heat capacity and IR spectroscopic properties from 0.1 to 0.2h represent binding of water at amide and carboxylate sites and presumably also other charged or polar sites. Saturation of these sites, if it is complete before significant covering of nonpolar elements, is expected to require^{8,9} about half the water needed for monolayer coverage, as is observed (0.2 versus 0.38h). The polar atoms are largely in the backbone and side-chain amide groups. The change in the amide I' band is a local effect produced by association of the amide group with water and not the result of changes in protein conformation¹⁰.

At 0.2–0.25h the carboxylate and carbonyl sites are saturated (Fig. 1a, b). The full coverage of the hydrogen-bonding sites is reflected in the differential diamagnetic susceptibility, which at this hydration level reaches the value for liquid water. At 0.25h the heat capacity rises (Fig. 1d) and then falls to the dilute solution value at 0.38h. This behaviour must be associated with coverage of the nonpolar elements of the protein surface, in view of the IR spectroscopic results that show saturation of hydrogen-bonding sites at lower hydration. The rise and fall in the heat capacity can be understood as a transition associated with covering of the least-strongly interacting regions of the surface. Statistical mechanical arguments indicate that a transition is expected at high coverage for adsorption of water on a heterogeneous surface¹¹. The magnitude of the 0.25h heat effect indicates that as many as 100 water molecules could be involved⁴.

The appearance of enzymatic activity at 0.2–0.25h (Fig. 1e) coincides with the saturation of the hydrogen-bonding sites and with the beginning of the coverage of the nonpolar regions of the surface. The catalytic activity is not simply reflecting water as a substrate in the hydrolytic reaction, because the dependence of the reaction velocity on water activity is tenth order. The new water arrangements on the protein surface that obtain above 0.25h may be required for catalysis. Spin-label studies⁵ show that these have greater motional freedom.

The apparent specific heat capacity shows no change between 0.38h and dilute solution. Because the heat capacity reflects all equilibria of non-zero enthalpy as well as the heat capacities of the components of the system, we regard this as sufficient evidence for the statement that the changes in thermodynamic properties associated with protein hydration are complete both for the protein and the solvent at 0.38h. This amount of water is barely sufficient to constitute monolayer coverage⁴ and corresponds to 300 molecules of water per protein molecule. Higher hydration is required to establish fully the kinetic properties for the dilute solution state.

We infer that between 0.1h and dilute solution, there can be no substantial change in protein structure. This is concluded from the monotonic behaviour of the IR spectroscopic properties of the amide and carboxylate groups between 0.07 and 0.25h, from the monotonic rise of the enzymatic activity observed above 0.2h, and from the constant apparent specific heat capacity observed from 0.38h to higher levels of hydration.

The following picture of the hydration process emerges from the findings and comments given above. The first water bound interacts with ionisable groups to produce proton redistribution. There is a clear transition in the hydrogen bonding between water and protein and water and water at 0.07h. This probably represents a change in the water molecule distribution about the protein surface, followed by an increase in freedom of the water–protein system. At 0.2–0.25h there is a major event in the hydration process, which is seen most clearly in the heat capacity and which coincides with the onset of enzymatic activity and the reaching of the final value of the response of the system to a magnetic field. Completion of monolayer coverage is at 0.38h, a value substantially greater than that for first observation of enzymatic activity. The protein with monolayer hydration shell must mesh simply with the bulk solvent.

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MATTERS ARISING

Age and significance of alluvium in the Windrush Valley

RECENTLY Hazelden and Jarvis¹ reported a radiocarbon date of $2,660 \pm 85$ yr BP for *in situ* roots in the gravel underlying the alluvium of the River Windrush, Oxfordshire. They suggest that this date represents the regional onset of clay deposition following forest clearance. This hypothesis discounts the possible role of channel migration which, although mentioned by the authors, was not discussed further.

Many rivers in southern and eastern England have gravel underlying the floodplain. Such rivers include the Trent, Nene, Great Ouse and Thames, together with many of their tributaries. The underlying gravels are continuous with those gravels lying about 1 m above the floodplain, forming the first or floodplain terrace. The majority of these first-terrace gravels seem to be middle-late Devensian in age², and they represent a phase of aggradation probably caused by increased sediment load during periglacial conditions. As sediment supply decreased, when periglacial conditions ended, downcutting occurred, giving rise to the wide channel now infilled by alluvium.

Deposition of fine-grained alluvium would be expected to have started as the ameliorating climate led to the formation of meandering rather than braided rivers. Dates from the base of the alluvium are highly variable, ranging from 10,000 to 2,500 yr BP (refs 3,4) and probably reflect the time at which the floodplain deposits were last reworked by a migrating meander rather than an ubiquitous change in conditions.

The deposits of the River Windrush^{1,5} are very similar to those found elsewhere and it therefore seems entirely plausible that an alder tree, growing through a pre-existing floodplain with roots in the underlying gravel, should have been eroded by a migrating meander at 2,660 yr BP with no change in sediment supply, climate or base level.

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HAZELDEN AND JARVIS REPLY—While this is a possible alternative explanation, it seems to us a less likely one. The truncation of the alder roots is associated with the gravel/clay interface; nowhere in the area have we seen the roots in the clay. Buried A horizons are common within the clay alluvium, and the sequence of deposits is the same over a large area of the floodplain, so it seems that extensive reworking by migrating meanders has not taken place. In view of this and the corroborative evidence from Farmoor we think our explanation more likely.

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Trace metals in remote Arctic snows: natural or anthropogenic?

NRIAGU¹ states that "It is encouraging that the ratios of the 1901–10 to the 1971–80 emissions of metals calculated from the data in Table 3 are consistent with the historical changes in rates of trace metal deposition observed in polar ice cores in the Northern Hemisphere". Some of the data cited^{2–4} in support cannot now be considered reliable, and we believe that the remaining data do not support his statement. The historical changes observed in polar ice cores appear much smaller than corresponding changes in worldwide anthropogenic emissions presented by Nriagu in Table 3.

The rate of trace metal deposition is a complex function of many variables, predominantly that of the deposition rate of snow. Reported deposition rates have been calculated from observed metal concentrations and from accumulation rates of snow which have not apparently

changed significantly during the period in question. There is no established theoretical or empirical relationship linking concentrations of metals in precipitation to those in air⁵, but most workers have assumed that the relative abundance of metals in snow reflects the composition of aerosols. Whilst this has been substantiated at the South Pole^{6,7}, recent work has revealed considerable differences in composition between Arctic aerosol and snow at Barrow, Alaska⁸.

Of the data cited by Nriagu, results obtained from a temperate glacier in Norway⁴ contain insufficient information about the local geology and analytical procedures, so that the abnormally high metal concentrations quoted must be treated with caution. The Dye-3 data² for recent snow are now believed^{3,9} to reflect local contamination from the nearby base manned since 1959. Using the data from Camp Century² and Station Milcent³, the ratios of metal concentrations in snow deposited during the periods pre-1900 and post-1960 have been calculated. These values, together with their standard deviations, are given in Table 1. Average concentrations in snow rather than ranges have been considered since the spread of extreme values may in part arise from seasonal variations¹⁰ and maximum values often represent contamination. The resulting ranges are so wide that no meaningful conclusion can be made.

Table 1 shows that the concentration ratios in snow are considerably smaller than the corresponding anthropogenic emission ratios. These discrepancies can be partially explained if the natural emissions quoted as most acceptable by Nriagu are added to the anthropogenic emission figures (last column of Table 1). There is even closer agreement if higher natural emission values (within the limits of values given by Nriagu and others¹¹) are chosen. It is to be expected that a higher proportion of trace elements of natural origin (particularly volcanogenic) will be transported long distances compared with emissions of anthropogenic origin, a major proportion of which return to Earth

Table 1 Comparison of global metal emission data with metal concentrations in Greenland snow

Metal	Anthropogenic emission ratio (from Nriagu's Table 3)	Camp Century snow concentration ratios ^{2*}	Station Milcent snow concentration ratios ^{3*}	Emission ratio including natural sources ¹
	1971–80	Post-1960 Pre-1900 $\pm \sigma$	Post-1960 Pre-1900 $\pm \sigma$	1971–80 1901–10
Cd	8.3	0.25 ± 0.27	1.0 ± 0.5	4.8
Cu	11.0	2.1 ± 1.6	—	3.2
Pb	9.1	—	3.2 ± 1.8	6.3
Zn	8.3	—	2.9 ± 1.65	4.5

* From averages of metal concentrations in snows.

close to their source¹². Volcanism has been invoked to account for the particularly high proportion of natural emissions in Antarctic snow⁶.

A substantial natural input of trace metals to Greenland snow has been reported by other workers¹³, and a large enrichment of heavy metals in comparison with crustal reference elements has been firmly demonstrated in both ancient and modern polar snows^{13,14}. Until the mechanisms of trace metal deposition are clearly understood, any attempts to assess temporal changes in atmospheric loadings from evidence in ice cores must be tentative.

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NRIAGU REPLIES—Landy et al. have misinterpreted my report¹. I was careful to say changes in rates of trace metal deposition rather than the ratios in trace metal concentrations in the ice layers. My statement clearly pertains to dF/dt (where F is metal flux rate at each ice layer and t is time), and is quite different from the linear relation, C_1/C_2 (C_1 , C_2 are metal concentrations of dated ice layers) assumed by Landy et al. The data tabulated by Landy et al. may thus bear no relationship whatsoever to the statement in my paper.

Landy et al. have dismissed the data of Jaworowski et al.² because “it contained

insufficient information about local geology and analytical procedures”. However, more than a page of this paper was devoted to materials and methods and included the cogent reference to the work on the characteristics of the Storebreen glacier. The high values of metals reported may not be due to sample contamination as such but may instead be related to deposition of long-range transported material from industrial centres of Europe (such as sulphate deposition and acid rains, see refs 3 and 4). In this sense, the Storebreen glacier probably contains a better record of changes in intensities of metal emissions in Europe.

The use of average concentrations of metals in ice layers deposited over a period of several decades or centuries (before 1900) in deriving the data listed by Landy et al. is misleading. It assumes that the rate of metal deposition was constant before AD 1900, which is contrary to the available evidence^{3,6}. I have calculated the ranges in the post-1960 to 1800–1900 ratios of reported trace metal concentrations in the ice fields of the Northern Hemisphere (Table A). The maximum and minimum values in reported metal concentrations during 1800–1900 and since 1960 have been used to derive the data listed. Obviously the ranges in the ratios are quite wide and the ratios obtained using my emission data are either within or fall near these ranges. This is ‘encouraging’ considering that the emission data are only order of magnitude estimates anyway.

The contention by Landy et al. that the trace metals in Arctic snow are derived mainly from natural sources is probably not valid. Rahn and his colleagues^{11,12} in fact have recently presented rather convincing evidence that the aerosols at widely dispersed Arctic sites of northern Norway, northern Greenland and Barrow, Alaska are strongly pollution-derived, particularly during the winter time. Before that, Chow and Earl¹³ used the isotope ratio method to show that most of the lead in Greenland glacier is of anthropogenic origin. Ancillary evidence in support of the anthropogenic origin of lead deposited in the snow fields of Northern Hemisphere are given by Settle and Patterson¹⁴. Note that the ratios of the present day deposition rates for Pb, Cd, Cu and Zn at Centrale, Greenland and at Dome C, Antarctica are

65, 16, 16 and 91 respectively¹⁵; the ratios of the Pb, Cd, Cu and Zn concentrations in present day surface snow at the two sites are 6.9, 1.6, 1.6 and 9.4 respectively¹⁵. Presumably, the much higher concentrations and deposition rates at the Arctic site vis-a-vis the Antarctic location is related to the fact that most of the anthropogenic emissions of metals occur in the Northern Hemisphere.

It should be emphasised, however, that thorough studies are needed on the deposition mechanisms and on the factors controlling the elemental composition of polar snow and ice before the metal concentrations in the ice layers can be related quantitatively to the atmospheric metal burdens. On this point, I completely agree with Landy et al.

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BOUSTRON COMMENTS—With regard to the above exchange of views I would like to comment on the available polar ice and snow historical records of the variations of the atmospheric concentrations of the five metals (Pb, Cd, Cu, Zn and Ni) discussed in Nriagu's paper¹, in the remote areas of the Northern Hemisphere during the past few centuries. Data such as those published for a temperate glacier in southern Norway² and quoted by Nriagu are not polar data, so that the only genuine polar historical records presently available for the Northern Hemisphere are those obtained in Greenland. Numerous data have been published on the trace metal content of the successive snow and ice layers deposited in the Greenland ice cap during the past few centuries. Unfortunately, many of these data are probably unreliable because they were plagued by severe contamination problems during field collection or (and) during laboratory analysis. It is very important then to estimate which of these data, if any, are reliable: this can be looked at by a detailed examination of the collection and analytical procedures described by the authors, with special reference to the use of high performance clean rooms and to

Table A Ranges in the post-1960 to 1800–1900 ratios of metal concentrations in the Arctic snow fields

Site	Cd	Cu	Pb	Zn
Milcent ⁷	1–2	—	1–7	1–8
Milcent ^{7,8}	<1–10	—	3–20	1–20
Dye-3 ⁹	—	—	9	—
Dye-3 ¹⁰	10–600	1–100	—	<1–40
Camp Century ¹⁰	<1	1–7	—	—
Camp Century ⁶	—	—	<1–>12	—
Jotunheimen Mountains ²	<1–68	—	<1–6	—

blank estimates both for field sampling and laboratory analysis.

Concerning the five metals discussed in Nriagu's paper, reasonably reliable Greenland data seem to be available only on the variations of the concentrations of Pb, Cd and Zn during the past 800 yr (refs 3, 4). On the other hand at present there seems to be no reliable Greenland data on the past variations of the concentrations of Cu and Ni. The reliable Pb, Cd and Zn data are shown in Fig. 1 for the past 300 yr; they do not include the Cd and Zn data published by Weiss *et al.*⁵, which have been shown recently^{4,6} to be erroneously high (by up to 2–3 orders of magnitude for Cd) probably because of severe contamination problems.

The interpretation of the variation profiles shown in Fig. 1 to assess man's global impact on the low atmosphere in the remote areas of the Northern Hemisphere is, however, still questionable for various reasons: (1) the scarce data available do not provide continuous variation profiles; (2) they have been obtained in several widely separated geographic sites; (3) some of the concentrations measured in the recent surface layers (post-1960 data) could be erroneously high because of local contamination problems in the vicinity of the Greenland stations; (4) there are still doubts on the efficiency of the decontamination procedures⁴ used to clean the deep ice core samples whose analysis has produced most of the pre-1900 available data; (5) for the years 1880–1960, which are the most interesting to assess man's impact, there are no Cd and Zn data, and only few Pb data; (6) the variation profiles could have been influenced by the arrival of volcanic aerosols emitted by the eruptions of the near Iceland volcanoes or by major eruptions which occurred in the world, such as Agung eruption (1963)^{7,8}; (7) the impurity content of Greenland snows could be influenced strongly by local crustal sources associated with the large ice free coastal areas of Greenland⁹; (8) there is no clear demonstration available that there are no chemical-fractionation processes at the air-snow interface in Greenland, such as the ones documented recently in Alaska⁹, which could lead to misleading interpretations of the snow and ice records in terms of atmospheric records.

Figure 1 suggests, however, that there has been no definite increase trend for Cd and Zn concentrations during the past 300 years. This is also the case for Pb before approximately 1940; after this date, a significant but limited increase of the concentrations of Pb seems to have occurred (about threefold increase). From the available data it seems, therefore, that the concentrations of Pb, Cd and Zn in Greenland snow and ice have not increased markedly during the past few centuries except probably for Pb since 1940, then suggesting that there has been no significant increase of the atmospheric

concentrations and then of the fallout fluxes F of these metals in the remote areas of the Northern Hemisphere since the beginning of the industrial revolution, except probably for Pb after 1940: the changes in anthropogenic emissions of these trace metals calculated by Nriagu¹ (from 1850–1900 to 1971–80: 19.5-fold increase for Pb, Cd and Zn) are then clearly much higher than the very limited historical changes in the fallout fluxes F of these metals observed in the remote polar areas of the Northern Hemisphere. (At a given polar site, the tropospheric fallout flux F of a metal during a given year is

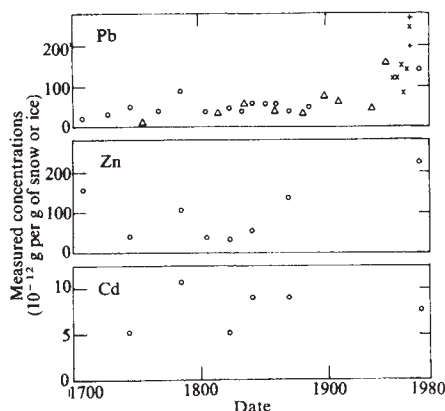


Fig. 1 Greenland: available reasonably reliable data on the concentrations of Pb, Zn and Cd in the successive snow and ice layers deposited since 1700. The different symbols correspond to separate sampling locations (O, Milcent; Δ, Camp Century³; +, 45 km ESE from Camp Century³; x, 80 km ESE from Camp Century³).

calculated by glaciologists as $F = CA$ where C refers to the mean measured concentration of the considered metal in the snow or ice layer deposited during this year and A is the annual snow accumulation during this year. In Greenland, A has been shown not to have changed markedly during the last centuries¹⁰.) Note that at present it seems impossible to derive from the scarce Greenland data shown in Fig. 1 any useful estimate of the time derivative dF/dt to which Nriagu claims to refer in his reply, and then to check whether the historical changes of dF/dt support the changes in anthropogenic emissions he calculated or not.

The available snow and ice Greenland data suggest strongly that the influence of human activity on the trace metals content of Greenland snow and ice, and therefore probably on the trace metals content of the low atmosphere in the remote areas of the Northern Hemisphere, is actually negligible for Cd and Zn and makes only a limited contribution for Pb: the high atmospheric enrichments observed presently for these metals in various remote areas in the Northern

Hemisphere^{11,12} are then very likely linked with natural processes such as volcanism.

However, it would be highly desirable to confirm and to complete the scarce Greenland data, through a cooperative analysis of new samples to be collected for this purpose, especially to get detailed continuous data on the trace metals content of the snow and ice layers deposited in Greenland during the past 100 yr.

Finally, I would like to comment on another point: the comparison¹¹ of the present day Pb, Cd, Zn and Cu concentrations measured in surface Greenland snows at Centrale⁶ with those measured in surface Antarctic snows at Dome C¹³. Present-day Greenland concentrations are higher than Antarctic concentrations probably principally because of the very different geographical locations and meteorological regimes of Greenland and Antarctica, and not only because of the fact that more than 90% of anthropogenic aerosols are emitted in the Northern Hemisphere¹⁴ as stated by Nriagu: Antarctica is indeed much more remote from any aerosol sources than Greenland, which is located near the North American continent, and is moreover protected from the influence of mid-latitude aerosol sources by the 50°S polar convergence.

From the present day surface concentrations at Dome C and at Centrale, and from the annual snow accumulation at these two sites, it is possible to get estimates of the present day fallout fluxes of Pb, Cd, Zn and Cu (ref. 11) in the central areas of Antarctica and Greenland: the fallout fluxes calculated at Centrale, Greenland are much higher than those obtained at Dome C, Antarctica, as pointed out by Nriagu. But this strong difference is certainly mainly related to the fact that the annual snow accumulation at Centrale ($34.3 \text{ g H}_2\text{O cm}^{-2} \text{ yr}^{-1}$) is one order of magnitude higher than the annual snow accumulation at Dome C ($3.5 \text{ g H}_2\text{O cm}^{-2} \text{ yr}^{-1}$).

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Polydipsia after intracranial injections—a property of NGF or a contaminant?

INTRACRANIAL injections of 2.5S NGF in adult rats have been shown to induce intense polydipsia¹. Subsequent studies from other authors confirmed this finding and, in addition, gave evidence of increased appetite for sodium solutions². The authors of this last article call attention to the similarity between these effects and those elicited by intracerebral renin injections. Previous work from this laboratory in collaboration with the Department of Pharmacology, University of Heidelberg³, showed that isorenin activity is present in 2.5S NGF preparations and is separated only after laborious additional purification steps. Thus, NGF prepared with the same method as that used for studies on induced polydipsia and sodium appetite still showed considerable amounts of renin-like activity. According to Cozzari *et al.*³ it was necessary to perform three further carboxymethyl-cellulose chromatographies after the usual procedure to prepare 2.5S NGF, in order to eliminate all renin-like activity previously detectable.

In view of the many effects which have been recently claimed to be caused by NGF intracranial injections^{4,5}, it seems essential that whenever high doses of NGF are needed to produce a given effect, it should be clearly established that the preparations are entirely free of contaminants. This precaution is of particular importance since NGF has been found to be tightly bound in mouse salivary extracts with other proteins endowed with enzymatic or other biological activities.

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LEWIS *ET AL.* REPLY—Levi-Montalcini is right to draw attention to the possibility that the effects of NGF on the central nervous system may be mediated by portions of the NGF complex other than the β subunit. As we pointed out in our report¹, the marked similarity between the effects of intracranial injections of 2.5S NGF and renin on thirst and sodium appetite suggested that the effects of 2.5S NGF might be mediated by the cerebral isorenin-angiotensin system. Activation of this system is an exceedingly potent stimulus to thirst² and sodium appetite³. We have since established that

7S NGF⁴ has similar effects on thirst and sodium appetite, and that both 2.5S⁵ and 7S NGF produced renin-like pressor responses when injected intravenously in intact and nephrectomised rats⁶. The doses of these NGF preparations needed to produce pressor responses are comparable to those used in the standard *in vitro* NGF bioassay⁷ and considerably less than the doses of NGF reported to induce growth in the sympathetic ganglia of newborn mice⁸. The pressor responses to 7S and 2.5S NGF were abolished by the angiotensin-converting enzyme inhibitor SQ 14,225, the angiotensin-receptor blocker saralasin, and NGF antiserum⁶. The same procedure also blocked the effects of these NGF preparations on thirst and sodium appetite⁹. Immunogenically pure β -NGF subunit¹⁰ from the mouse submandibular gland was devoid of pressor activity and had a much smaller and more variable effect on thirst and sodium appetite than other preparations of NGF⁶.

Our demonstration that NGF-induced thirst and sodium appetite are mediated through the formation of angiotensin II (AII) raises the possibility that other effects of NGF preparations are similarly mediated. For example, we have recently found that intracranial administration of renin, like 2.5S^{11,12} and 7S NGF, results in a marked increase in the activity of ornithine decarboxylase (ODC) in brain and liver⁶. These increases in ODC activity in response to renin and NGF are blocked by pretreatment with SQ 14,225 and are therefore dependent on the formation of AII⁶. The AII-dependent induction of ODC in the liver following NGF administration is due to activation of the pituitary-adrenal axis, as the induction is blocked by either hypophysectomy or adrenalectomy¹². Otten *et al.*¹³ recently reported that systemic injection of 2.5S NGF, the purity of which was controlled by SDS-polyacrylamide gel electrophoresis¹⁴, caused a rapid increase in plasma ACTH and corticosterone levels. However, systemic or central administration of AII also causes an increase in plasma ACTH and corticosteroid levels^{15–17}. Therefore, activation of the pituitary-adrenal axis following NGF administration may be due to the presence of a renin-like enzyme in the NGF.

Our findings complement those of Cozzari *et al.*¹⁸, who reported that NGF preparations from mouse submandibular gland are associated with a renin-like enzyme that can only be removed after much additional purification. Whether this renin-like enzyme or other enzymes^{19,20} should be regarded as integral components of mouse submandibular gland NGF complexes has not been resolved. However, unless renin-free preparations of NGF are used, the use of angiotensin antagonists such as SQ 14,225 and saralasin should be considered

obligatory, particularly when a biological effect attributed to NGF is known to be produced by renin or AII.

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Evolution by gene duplication in insecticide-resistant *Myzus persicae*

THE simple model of “a succession of tandem duplications of the structural gene”, proposed by Devonshire and Sawicki¹, cannot account as it stands for the apparent regularity of the variation in enzymatic activity of the organophosphorus (Op) insecticide resistance associated esterase (E4¹ or RAE²) observed in specimens of the peach potato aphid, *Myzus persicae*, from different laboratory stock clones, because the esterase does not have the same electrophoretic mobility or phenotype in all the clones compared.

The enzyme from the predominant Op resistant clone in British field populations³ (E4(MS1G) or RAE(+)²) stains after electrophoresis in two close but distinct bands, the rear band being fainter and staining more slowly². Aphids with this electrophoretic phenotype, but with higher enzymatic activity on the gel, have also been collected in the field², and these could have arisen by duplication of the E4(MS1G) gene, although other explanations are also possible, for example, that they are homozygous for E4(MS1G), or that they are a mutant in which E4(MS1G) has become more resistant to denaturation in gel assay

conditions. But the high activity enzyme in the laboratory clone French R and the very high activity enzyme in the highly Op-resistant glasshouse clone PirR (electrophoretically identical to that in the highly resistant clones from northern England and western Scotland⁴) both stain after electrophoresis in a single band with an electrophoretic mobility slightly but distinctly different from E4(MS1G) and from each other.

Because of this electrophoretic distinctiveness it is unlikely that either of these high activity mutant esterases could have arisen by a duplication of the E4(MS1G) gene, or that the higher activity esterase (E4(PirR)) could have arisen by a duplication of the gene for the lower (E4(French R)). A more reasonable explanation would be that E4(MS1G), E4(French R) and E4(PirR) arose independently in separate mutations in different Op-susceptible aphids.

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DEVONSHIRE REPLIES—The simple model of “a succession of tandem duplications of the structural gene” is based on the measurement of insecticide hydrolysis¹, and not simply on the subjective assessment of electrophoresis gels stained for naphthyl acetate-hydrolysing enzymes. The enzyme, E4, has been shown to be identical in susceptible and resistant aphids, and differences in activity are a direct consequence of the production of more of the same enzyme¹.

Esterase E5 (which probably corresponds to ‘the satellite’² to E4³) is present in MS1G and French R, but not in the other five clones examined¹ and seems to occur only in untranslocated variants. Any association of this band with E4 is speculative, and our studies have shown that it plays no part in insecticide hydrolysis. Baker suggests that the six specimens from two sites, in which “the electrophoretic band (E4) appeared³ to be slightly retarded in mobility and without a satellite (E5?)” are associated with glasshouses. We have never detected such decreased mobility of E4 in slightly resistant variants among the several thousand insects examined from the field and glasshouses throughout the UK. The biochemical and toxicological significance of this putative electromorph has not been investigated, and it has little bearing on our conclusions. Although very resistant aphids (with 32 or 64 times as much enzyme) appear to have E4 with slightly lower mobility, on dilution the enzyme reverts to the characteristic mobility either when run alone or when coelec-

trophoresed with E4 from susceptible insects. It is therefore an artefact of the electrophoresis probably arising from the larger amount of protein present.

In view of the convincing biochemical evidence, and lack of ‘electrophoretic distinctiveness’ of E4 in the seven variants examined, we believe gene duplication to be the most likely cause of the overproduction of E4 by resistant aphids. This, however, does not preclude hitherto undiscovered mutations at this structural locus.

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Spider feeding behaviour optimises dietary essential amino acid composition

GREENSTONE¹ showed that dietary mixing occurs in the lycosid *Pardosa ramulosa* (McCook) and claimed that the spiders optimise their intake of essential amino acids. He took the optimal proportions of amino acids to be those present in the spiders and estimated the ingested nutrients from the amino acid composition of ‘appropriate extracts’ of the prey. I suggest that the extracts he used were inappropriate so that the data cannot be used to test the hypothesis that they forage optimally for essential amino acids.

The appropriate extract used by Greenstone was prey haemolymph “because spiders do not consume their prey intact and therefore do not ingest skeletal material” (see Table 2 of ref. 1). However, spiders pour digestive fluids onto their prey and ingest the fluid products and the haemolymph but discard the cuticle. In *Tegenaria atrica* Koch these digestive fluids contain a wide range of proteolytic enzymes as well as esterases and carbohydrases². A similar range of digestive enzymes must be expected in all spiders.

Except for the haemolymph, the distribution of amino acids in insects is poorly known but the information available shows no correlation between the amino acid composition of insect protein and that in the free pool amino acids³. In addition the amino acid composition varies between tissues as well as between sexes⁴. Consequently the amino acid composition of ingested material will reflect that of both the haemolymph and of those tissues digested externally. The proportion of each tissue digested will vary because spiders consume different amounts of individual prey items according to, *inter alia*, disturbance, prey type, and hunger and/or prey density. The relationship between the amino acid content of prey haemolymph and the food ingested needs

to be established before the hypothesis can be tested.

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GREENSTONE REPLIES—As Humphreys intimates, the most appropriate extract for the dietary studies is not an easily extractable fraction, but rather the difference between the total amino acid composition of the insect and that which remains in the carcass after the spider has fed upon it. At the time the amino acid studies were begun it was not possible to initiate a feeding study. Large stocks of the insects, however, were on hand, from which two types of extracts were potentially available: haemolymph alone, which might underestimate the spiders’ amino acid consumptions, and macerates of whole insects, which might overestimate them. I chose to risk underestimation because the spiders’ evolutionary history has predisposed them to having low prey utilisation efficiencies.

Pardosa species are small and live in open habitats¹; this exposes them to heavy density-independent mortality. *P. ramulosa* spiderlings generally have a high ballooning frequency (my unpublished data), which is a correlate of habitat instability^{2,3}. Open and unstable habitats are r-selecting, and among the expected traits of r-strategists is low food utilisation efficiency⁴. *Pardosa* species have been shown to have lower prey utilisation efficiencies⁵, and jumping spiders to feed for less time on each prey item⁶, when food is abundant, as was the case in the study area during my field work⁷. These facts suggested that the spiders would be more apt to take only haemolymph than to consume all of the extractable amino acids.

The use of haemolymph has the additional advantage that material from a large random sample of an insect population can be pooled, thereby averaging out some of the age and sex differences in amino acid composition.

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BOOK REVIEWS

Food for thought

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BEFORE the 1970s most neurochemists (stimulated by current ideas on biochemical regulation) were probably more interested in brain enzymes than in the substrates they acted on. Consequently, the availability of these substrates to the brain was given relatively little attention. Also, it was thought that, except in severe malnutrition, the brain controlled its nutritional intake so that this was appropriate to its needs. But in the past decade emphasis has shifted, especially with regard to neurotransmitter synthesis. The change began with studies on how brain synthesis of the transmitter 5-hydroxytryptamine (5HT) is controlled. Tryptophan hydroxylase, one of the two enzymes required for 5HT synthesis, is rate-limiting but normally well below saturation with its substrate, tryptophan. This is an essential amino acid, mammals are completely dependent on dietary sources and it is found that normal dietary changes of tryptophan availability to the brain can affect how much of the transmitter is made there.

These findings have led to an explosion of research activity in which Wurtman and his group have played a major role. This interest is strongly reflected in the series so that many chapters describe how dietary constituents get to the brain and their effects on transmitter synthesis therein. Indeed, the whole of Vol. 5 derives from a symposium on the effect of choline supply on brain acetylcholine synthesis. Apart from this, five chapters, distributed between Vols 1, 3 and 4, deal wholly or in major part with dietary or other extracerebral influences on transmitter synthesis. Thus, in Vol. 1, Pardridge describes how amino acid availability to the brain is regulated and Ordonez similarly discusses folic acid derivatives and choline. Volume 3 contains a chapter by Growdon on dietary neurotransmitter precursors and their use in the treatment of brain disease. Another chapter by Sourkes is on other nutritional factors that may affect transmitter synthesis. Volume 4 includes a chapter by Guroff on how inborn errors of extracerebral metabolism of amino acids and other substances alter the nutrition of the brain.

Nutrition and the Brain. Edited by R.J. Wurtman and J.J. Wurtman. Five volumes. (Raven: New York.) Vol. 1. *Determinants of the Availability of Nutrients to the Brain*, pp.336, 1977, \$29. Vol. 2. *Control of Feeding Behavior and Biology of the Brain in Protein-calorie Malnutrition*, pp.323, 1977, \$29. Vol. 3. *Disorders of Eating. Nutrients in Treatment of Brain Disease*, pp.314, 1979, \$29. Vol. 4. *Toxic Effects of Food Constituents on the Brain*, pp.232, 1979, \$23. Vol. 5. *Choline and Lethicin in Brain Disorders* (edited by A. Barbeau, J.H. Growdon and R.J. Wurtman), pp.474, 1979, \$42.

Another major area is covered in Vol. 2 and the rest of Vol. 3. These chapters are on normal and pathological aspects of feeding: control of eating (Vol. 2, Lytle); metabolism in obesity and anorexia nervosa (Vol. 3, Cahill *et al.*); medical aspects of these disorders (Vol. 3, Bruch); a critical analysis of claims for megavitamin therapy in mental disease (Vol. 3, Lipton *et al.*). Most of this material is on effects of disturbances of volition towards eating. The brain obviously has a causal role in these disturbances although little is at present known about its nature. The rest of Vol. 2 is on protein-calorie malnutrition — a primarily involuntary disorder. Here the important questions concern effects of the reduced food intake on the human brain, their reversibility and their behavioural consequences. As Shoemaker and Bloom point out in their chapter on the effect of undernutrition on brain morphology, early undernutrition might conceivably affect subsequent behaviour and other brain functions by numerous mechanisms. While present evidence for specific mechanisms may well be unclear this does not diminish the enormous potential social importance of research in this area. Other chapters in Vol. 2 are on biochemical (Nowak and Munro) and behavioural (Pollitt and Thomson) effects of protein-calorie malnutrition. Background material on the dietary patterns of numerous human societies and of non-human primates is given in Vol. 1 by Gaulin and Konner.

Tables 7–9 are particularly fascinating.

Volume 4 is largely on toxic effects of food constituents, i.e. food additives and hyperkinesia (Lipton *et al.*), glutamic acid (Garattini), peptides (Zioudrou and Klee), alcohol (Tabakoff *et al.*). These effects range from the obviously important but imperfectly understood (alcohol) to the probably unimportant (glutamic acid), although it is necessary to keep in mind the conclusion of the chapter on hyperkinesia that there is probably no food or drug without some toxicity to some individuals.

Volume 5 is substantially different from the other volumes, which each contain four to six chapters, as it contains 38 contributions to a symposium. Topics include choline, lecithin and acetylcholine metabolism, and the pharmacology of the cholinergic neurone. The main focus is on the extent to which functional brain cholinergic activity is affected by dietary supplements of choline and lecithin and whether these compounds can be used to treat various disorders of the central nervous system. There seems to be some evidence that large dietary supplements may alleviate tardive dyskinesia but not other dyskinetic states such as Huntington's chorea and dopa dyskinesia. Patients with presenile dementia, though they have a major defect in brain acetylcholine synthesis, show disappointingly little response. In general, the therapeutic trials described were of a preliminary nature. Neither of the studies on tardive dyskinesia was done 'blind'. The chapters by Ansell and Spanner, Haubrich *et al.* and Karczmar are particularly useful, as they provide a biochemical and pharmacological background for assessing the (on the whole less substantial) clinical papers which take up much of the volume.

The series in general is strongly recommended. It is attractively produced and well indexed. The overall clarity of presentation must indicate much hard editorial work. One may expect these volumes to be a major influence on future research in their field. □

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Methods of polarographic analysis

Emil Paleček

Polarography of Molecules of Biological Significance. Edited by W. Franklin Smyth. Pp.328. (Academic: London and New York, 1979.) £22.60, \$52.

THE sensitivity of polarographic (voltammetric) determinations has greatly increased in the past 10-15 years. While the limits of detection of organic and inorganic substances reached by classical d.c. polarographic methods were usually around 10^{-5} M, using modern differential pulse anodic stripping voltammetry, inorganic substances can be determined at concentrations down to 10^{-10} M. Cathodic stripping voltammetry is becoming a very useful tool in the analysis of certain organic compounds at concentrations as low as 10^{-8} – 10^{-9} M; and differential (derivative) pulse polarography operates at concentrations of 10^{-6} – 10^{-8} M for both organic and inorganic substances. This remarkable gain in sensitivity, together with the commercial availability of reliable polarographic analysers, has resulted in a steady increase in the number of analysts using modern polarographic (voltammetric) methods. This process, sometimes called the 'renaissance of polarography', is, however, not yet accompanied by the predictable abundance of books concerning the theory and application of modern polarographic methods. Smyth's book is thus a valuable acquisition for analytical chemistry libraries.

The first two chapters of this book describe unit processes in polarographic (voltammetric) analysis of organic substances such as choice of method, preparation of the sample, selection of a proper supporting electrolyte, cell and electrodes, derivatization and complexation procedures, and so on. Further chapters deal with the application of polarographic methods in pharmacy and pharmacology, basic medical and environmental sciences, and agriculture. Polarographic determinations of various groups of substances are in general compared with determinations by means of other methods; and the advantages of the polarographic analysis of certain types of compounds are stressed. It is shown that differential pulse polarography is highly suitable for the determination of pharmaceutical products and other substances containing nitrogroups; and sulphur-containing substances (many of them representing trace foreign materials which pollute the environment) can be determined at low concentrations by cathodic stripping voltammetry.

The book is well-written; it has not,

however, managed to escape entirely without errors; for example, in the paragraph on purine and its derivatives (Chapter 5) it is stated that pyrimidine and cytosine are oxidizable at graphite and mercury electrodes. It follows from the cited paper (G. Dryhurst and P. J. Elving) that both substances are non-oxidizable. In spite of the fact that a relatively extensive literature on polarography of nucleic acids exists, only two original papers are cited in Chapter 6, one of them incorrectly; review articles concerning this topic are

completely ignored.

Minor errors do not, however, substantially diminish the significance of this book, which can be confidently recommended to all analytical chemists dealing with determinations of biologically important organic substances, and especially to those of them who have come in contact with polarography in the past. □

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Interstellar dust

D.E. Brownlee

Cosmic Dust: Its Impact on Astronomy. By Peter G. Martin. Pp.266. (Clarendon/Oxford University Press: Oxford, 1979.) £9.

PETER MARTIN'S *Cosmic Dust: Its Impact on Astronomy* is not an encyclopaedia of what is known about extraterrestrial dust. It does not cover Moon dust, Mars dust, comet dust, Venusian smog or plumes from Io's volcanoes. It is a book which concentrates on star dust and is a comprehensive coverage of current information and ideas concerning dust in the interstellar medium. Interstellar dust is a basic form of matter in galaxies and is the common form of most elements heavier than helium that are not currently residing in stars. Interstellar grains are produced in gaseous regions surrounding stars; they experience an evolutionary existence as tiny astronomical bodies and are eventually destroyed by super novae shocks or interactions with stars. They are somewhat of a nuisance, as they totally obscure most of our Galaxy in visual wavelengths and cause extinction, reddening and polarization in observable objects. However, as Dr Martin enthusiastically points out, dust grains are fascinating bodies important to a variety of disciplines in astronomy.

The book's first five chapters deal with the interaction of dust and radiation in the interstellar medium. These chapters cover fundamental theory as well as up-to-date observational and laboratory data. Topics include radiation transfer, calculation and laboratory measurement of particle cross-sections, extinction of optical and X-ray wavelengths, polarization, and scattering. The fifth chapter deals with the properties and modelling of reflection nebulae. In addition to providing the reader with a good background on the interaction of light and dust, the author also interprets existing observations in terms of limitations they place on the nature and distribution of interstellar dust.

Chapters six and seven discuss the theory and observation of thermal emission from grains. This includes far-infrared from the

Galactic plane and the properties and structure of circumstellar dust shells. Chapters eight and nine deal with the distribution of dust in the Galaxy, the nature of dust clouds and interactions of dust with the interstellar environment. Specific topics include radiation pressure, spin, charge, sputtering, molecule formation, alignment and dust in dark clouds, and H II regions, planetary nebulae and the Solar System. The coverage of most of these topics is somewhat introductory and does not go into considerable depth. In chapter ten the significance of spectral signatures such as the 2200 Å and 10 µm features are discussed as indicators of grain composition. Also discussed are the importance of cosmic abundances and abundances in interstellar gas as constraints on grain composition. Chapter eleven reviews the evolutionary processes and timescales experienced by dust. Basically this chapter is concerned with the formation of refractory cores and volatile mantles and their stability in changing interstellar environments. The final chapter contains a brief section on dust in other galaxies and an intriguing discussion about properties and possible observations of dust between galaxies.

In the preface the author states a goal to give the reader basic tools and to discuss concepts underlying current research. This he does quite well. A careful reading of this book will provide the reader with a broad-based background in both the theory and current state of observation of interstellar grains. In general the book can be highly recommended, although there are some minor drawbacks. The organization is such that certain topics are diffused through widely separated parts of the book. It sometimes is difficult to answer a specific question without reading most of the book. Although it provides a good background, this is not a handy source book for quick reference. The most serious drawback in the opinion of this reviewer is the lack of formal references. At the end of each chapter is a list of "further reading" but these are not true references that refer to specific points in the text. This continually raises a question of credibility. The reader is apparently expected to accept the written word as dogma. The lack of conventional references also makes it difficult for the

reader to go into a specific subject in more depth than covered in the text. Annoying but less serious problems are with the figures. Most of the line drawings are excellent and were printed in a readable standard format. A small number of drawings, however, are direct computer plots with tiny difficult-to-read labels and needless excess zeros behind the decimal point. Many of these are multifunction plots where individual lines are very difficult to distinguish from each other. Only a few plots are really terrible but they are certainly not for the farsighted. Some

of the letters are less than $800\mu\text{m} \times 500\mu\text{m}$, some of the plotted lines are actually less than a $100\mu\text{m}$ in width, and in places both plotted lines and figure borders totally disappear. The continuous tone photographs have poor tonal quality similar to newsprint.

Cosmic Dust is a comprehensive up-to-date book that will be of significant value to anyone working in fields involving interstellar grains. It contains a good coverage of the theory, observations and current state of understanding of dust in the interstellar medium. For someone just

getting into this interdisciplinary field it contains a wealth of suggestions for important future work. For the corner librarian this book will be a headache. Although *Cosmic Dust* is the first *Cosmic Dust* devoted entirely to interstellar dust, it is the third book with a jacket label of this title published in the past 15 years. □

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Forest ecosystem

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Pattern and Process in a Forested Ecosystem: Disturbance, Development and the Steady State Based on the Hubbard Brook Ecosystem Study. By F.H. Bormann and G.E. Likens. Pp.253. (Springer: Berlin, Heidelberg and New York, 1979.) DM42, \$23.10.

As the sub-title indicates, this book describes the work of the Hubbard Brook Ecosystem Study in northern New England. The authors' objective is to provide an integrated picture of the structure, function and development of an area of northern hardwood forest and to set their data against current concepts of ecosystem dynamics. In effect, this book is one of the most complete accounts of secondary woodland succession following clear felling so far to be produced.

The authors propose a four-phase biomass accumulation model of woodland development of reorganization, aggradation, transition and steady state. This differs from the familiar smooth-curve biomass accumulation model of Odum in that the initial 'reorganization' phase of up to 15 years is a period of net loss in biomass when total ecosystem respiration exceeds GPP, as is also the transition phase which precedes the final 'steady state', when total biomass oscillates about a mean. Most of the book is inevitably concerned with a discussion of the quantitative characteristics of the 'reorganization' and 'aggradation' phases as these have been the subject of the long-term study. Here can be found a wealth of data on biomass accumulation, productivity, hydrological and mineral cycles, species diversity and evolutionary strategies. The discussion of the 'transition' and 'steady-state' phases is necessarily speculative as no examples of primeval forest survive in New England and the authors estimate that in the absence of external catastrophic events it takes about 300 years for the steady-state phase to be reached by natural developmental processes.

Many authors in the last twenty-five years have questioned the classical Clementsian view of 'climax' vegetation, insisting that it represents no more than an unrealizable abstraction. This is based on the assertion that natural succession is inevitably truncated somewhere in the aggradation phase (of the present authors) by cyclical catastrophic events such as fire or wind-blow. This issue is fully examined in the light of the available historical and ecological evidence, and the authors conclude that so far as their study area is concerned the fire-rotation periods are far longer than would be needed to prevent natural development reaching the steady-

state phase and that climax forest would indeed have been a reality in pre-settlement times.

Throughout the book there are undertones of a Clementsian 'supra-organism' philosophy but these are not permitted to become unduly obtrusive. The authors freely acknowledge that much more is owed to A.S. Watt's concepts of pattern and process in vegetation, an approach that will meet with a sympathetic hearing from most British ecologists. □

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Sediment studies

R.C. Selley

Coastal Sedimentation. Edited by D.J.P. Swift and H.D. Palmer. Pp.339. (Dowden, Hutchinson and Ross: New York. Distributed by Academic: New York and London, 1979.) \$34, £22.50.

In the words of Professor Fairbridge, Editor of the *Benchmark Papers in Geology*, the philosophy behind the series is "one of collection, sifting and rediffusion". The basic concept is to republish selected seminal papers on a chosen topic in their original form (with minor excision) enveloped in editorial comment.

This is designed to overcome the problems of a scientist trying to delve into original literature only to find that the important references are diffused through many journals, or that, in the case of the newer centres of learning, runs of journals are of too short a span to contain vintage papers.

The first of these problems is particularly acute in the field of coastal sedimentation which is reviewed in this particular benchmark volume. This is a topic which is of interest to a wide range of scientists, engineers and even ecologists and conservationists. These range from geographers, geologists, civil and

hydraulic engineers to marine ecologists and sailors. Thus, it is not surprising to find that the papers in this volume are reprinted from periodicals as diverse as the *Journal of Waterways and Harbors Division of the American Society of Engineers*, the *Bulletin of the Georgia (USA or USSR) Academy of Sciences*, the *Bulletin of the American Association of Petroleum Geologists*, the *Beach Erosion Board of the US Army Corps of Engineers*, and a symposium volume published by the National University of Mexico. Few scientists can claim to have such eclectic interests as to monitor all these publications. They must be too busy reading to do any work if they can.

The book reprints a total of twenty papers which have been arranged in four groups. Each group is preceded by several pages of comment and introduction by the editors.

The papers in Part I are concerned with the coastal equilibrium profile. They begin with a bowdlerized version of Fenneman's classic paper of 1902, twenty pages having been omitted from the original thirty-two. This is followed by several papers written by US geologists in the nineteen-sixties. Part II is concerned with coastal deposits, reprinting five papers describing certain North American beaches and shelves. Part III is headed "Studies of Fluid Motion" and reprints five papers ranging from "Bottom Currents during Hurricane Camille" to "Wind-Driven and

Thermohaline Circulation over the Continental Shelves". Part IV contains four papers on the theme "Studies of Substrate Response". These present observations on the erosion, transportation and deposition of sand in selected coastal areas. The book concludes with author and topic indexes.

From the preceding account it is clear that the titles of this volume should perhaps not have been so catholic as *Coastal Sedimentation* but rather 'North American Sandy Coasts'. No two scientists would produce the same top-twenty list of significant papers on coastal sedimentation. Nevertheless, the concentration on sandy coasts

of the USA is surprising. Only two papers deal with non-US coasts, one in Mexico, the other in the North Sea. There are no papers on carbonate coasts, either of such classic areas as the Bahamas or the Persian Gulf. Neither are there any papers on deltas, or on muddy coasts, such as the tidal flats of the North Sea made famous by Dutch and German workers.

A review of previous benchmark volumes shows that coastal sedimentation has already been done in various disguises — No.3, Spits and Bars, No.9, Barrier Islands, No.30, Holocene Tidal Sedimentation, and No.39, Beach Processes and Coastal Hydrodynamics. Perhaps some of

the omissions noted in this book have appeared previously. A volume entitled 'Benchmark Papers of Benchmark Papers on Coastal Sedimentation' is perhaps now overdue.

It is hard to see this particular book appealing to many individual scientists. Perhaps only the newest and wealthiest of libraries may consider purchasing it. Since the text has been prepared by photographically copying the original papers, some in typescript, the cost of £19.15 seems rather high, perhaps even a benchmark. □

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Glucocorticoids at work

G. S. Boyd

Glucocorticoid Hormone Action. Edited by J. D. Baxter and G. G. Rousseau. Pp.638. (Springer: Berlin, Heidelberg and New York, 1979.) DM 118, \$64.90.

THERE is interest in the mode of action of the steroid hormones in attempts to discover at the molecular level how these hormones work. Investigation of the way in which sex hormones act is simpler since it is possible to identify specific target tissues. However, the glucocorticoids present a difficult challenge because these steroids have such widespread effects, and the tissue response to the hormone depends upon the nature of the tissue.

The late Gordon Tomkins played a significant part in attempts to discover how the glucocorticoids work, and it is fitting that this volume should be dedicated to the memory of this brilliant researcher.

The book is a series of short essays on various phases of the possible mode of action of glucocorticoids. The editors have contributed an introductory chapter, various other chapters and a concluding generalizing chapter updating some of Tomkins' concepts and placing these views in a modern setting. The volume has over 600 pages and is divided into 34 chapters so that any one contribution is fairly short. The contributors appear to have been adequately briefed by the editors because there is a reasonable degree of continuity throughout this multi-author review. The book contains accounts of the transport and uptake of corticoids by cells. Various aspects of the association of the hormones with the cytosolic receptor molecules are expounded, the structure-activity relationships are explored, and the translocation into the nucleus and the nature of the nuclear binding of the glucocorticoids are considered. The grey areas on the activation of the genetic

apparatus in the nucleus are critically reviewed and the role of the glucocorticoids as important factors involved in the induction of tyrosine aminotransferase and tryptophan oxygenase is considered. It seems a pity that the chapter on tryptophan oxygenase is one of the shortest in the book. In separate chapters various authors deal with the mode of action of corticoids on fibroblasts, thymocytes, leukaemic cells, pituitary cells and leukocytes. There are also contributions on the role of glucocorticoids in glycogen metabolism,

lipid metabolism, insulin action and the stability of lysosomes. The editors have made a bold attempt to obtain coverage of as many aspects of corticoid action as possible in this sizeable book. Naturally this has resulted in a degree of overlap in places. Nevertheless this is a good book which will be a useful source to all who wish to have ready access to information on the action of the glucocorticoids up to about 1977-1978. □

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Collision theory

A. D. Buckingham

Atom-Molecule Collision Theory. Edited by R. B. Bernstein. Pp.779. (Plenum: London and New York, 1979.) \$57.50.

IN the last 20 years there has been an enormous growth of interest in the study of molecular collisions by beams techniques. In 1977 the American Chemical Society was able to arrange a symposium entitled "State-to-State Chemistry". The advances of electronic and vacuum instrumentation have produced a wealth of information on atomic and molecular cross-sections covering elastic, inelastic and reactive scattering. This volume is intended as a guide to scattering theory for experimentalists in the field of molecular collisions. It is restricted to atom-molecule collisions, for which the theory is at present tractable.

The first chapter is by Bernstein himself. It provides an interesting and informative overview of the development of the beams technique and of the current state of experiment and theory. The second chapter, by H. F. Schaefer III, reviews the *ab initio* computation of potential energy surfaces; the third, by P. J. Kuntz, is on semiempirical potential surfaces for collision theory. The analysis of elastic

scattering is discussed in great detail by H. Pauly for central forces, and by S. Stolte and J. Reuss for non-central forces. Inelastic scattering theory is covered in chapters by J. C. Light, D. J. Kouri, D. Secrest, M. D. Pattengill and W. R. Gentry. Non-adiabatic electronic transitions are described by M. S. Child, and reactive scattering by J. C. Light, R. E. Wyatt, D. G. Truhlar, J. T. Muckerman and D. A. Dixon. There are chapters on collision-induced dissociation by D. J. Diestler and P. J. Kuntz, and the final contribution is by R. D. Levine and J. L. Kinsey on the application of information theory to molecular collisions. There are 22 chapters and author and subject indexes, making up the 779 pages.

The book has some of the usual problems associated with a multi-author volume; however these are not grave, and the overlap and the changes in notation are not really a serious disadvantage. As compensation the spread of authorship, including many distinguished contributors, has provided a breadth of coverage that no single writer could have achieved. The book is highly recommended to those for whom it was intended — experimentalists in the field of molecular scattering. It will also be valuable to a wider group of physicists and chemists interested in the dynamics of molecular collisions. □

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